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Hypothermia in Stroke Therapy: Systemic versus Local Application

Mitchell Huber, Hong Lian Duan, Ankush Chandra, Fengwu Li, Longfei Wu, Longfei Guan, Xiaokun Geng and Yuchuan Ding

Abstract
Presently, there are no effective, widely applicable therapies for ischemic stroke. There is strong clinical evidence for the neuroprotective benefits of hypothermia, and surface-cooling methods have been utilized for decades in the treatment of cerebral ischemia during cardiac arrest, but complications with hypothermia induction have hindered its clinical acceptance in ischemic stroke therapy. Recently, the microcatheter-based local endovascular infusion (LEVI) of cold saline directly to the infarct site has been proposed as a solution to the drawbacks of surface cooling. The safety and efficacy of LEVI in rat models have been established, and implementation in larger animals has been similarly encouraging. A recent pilot study even established the safety of LEVI in humans. This review seeks to outline the major research on LEVI, discusses the mechanisms that mediate its superior neuroprotection over surface and systemic cooling, and identifies areas that warrant further investigation. While LEVI features improvements on surface cooling, its core mechanisms of neuroprotection are still largely shared with therapeutic hypothermia in general. As such, the mechanisms of hypothermia-based neuroprotection are discussed as well.

Keywords: local endovascular infusion, therapeutic hypothermia, ischemic stroke therapy, neuroprotection, microcatheter

1. Introduction
Ischemic stroke is the leading cause of death and disability worldwide, yet effective treatment is limited. Despite considerable research efforts, intravenous (IV) thrombolysis with recombinant tissue plasminogen activator (rt-PA) within the first 4.5 h of symptom onset remains...
the only proven acute therapy for ischemic stroke [1]. Outside of the treatment window, rt-PA fails to be an option, and given that only 25.4% of stroke patients arrive to the hospital within 3 h of symptom onset, a significant minority of patients are even eligible to receive rt-PA [2]. Thus, alternative treatment strategies for ischemic stroke are urgently needed. Although over a thousand drugs and nonpharmacological strategies have been tested for neuroprotective ability in acute stroke as of 2003, none have proven effective and applicable enough for widespread clinical acceptance [3]. However, hypothermia has prevailed as a promising therapeutic option for stroke patients. In fact, hypothermia is the only neuroprotective approach found thus far whose efficacy has been experimentally demonstrated in a randomized controlled clinical trial [4]. The neuroprotective benefits of hypothermia have been utilized for decades in the treatment of global cerebral ischemia following cardiac arrest and for hypoxic-ischemic encephalopathy in newborns, but its use in stroke therapy has garnered attention only in recent years [4, 5].

Hypothermia has been consistently shown to reduce infarct volumes and improve functional outcomes in animal models of focal cerebral ischemia. In a meta-analysis, the use of hypothermia in animal models of ischemic stroke was shown to reduce infarct volumes by 44% on average [6]. Given the robust neuroprotective effects of therapeutic hypothermia (TH) in animal models of temporary artery occlusion, studies are being conducted at an increasing rate to empirically establish hypothermia as a high-yield front-line stroke therapy.

1.1. Degrees of hypothermia

Therapeutic hypothermia is defined as the deliberate reduction of core body temperature for therapeutic benefit [7]. While there is no exact consensus on the optimal degree of cooling, several studies have found cooling at 33°C to be most effective [7–10]. The vast majority of investigations on the topic feature a mild to moderate degree of cooling, with very few venturing into moderate-deep to deep hypothermia (Table 1). In fact, temperature depressions to such an extent have been reported to primarily provide negative consequences [9]. For this review, therapeutic hypothermia refers to mild-moderate hypothermia, unless otherwise specified.

<table>
<thead>
<tr>
<th>Degree of hypothermia</th>
<th>Mild</th>
<th>Moderate</th>
<th>Moderate-deep</th>
<th>Deep</th>
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<tr>
<td>Body temperature (°C)</td>
<td>35.9–34</td>
<td>33.9–32</td>
<td>31.9–30</td>
<td>&lt;30</td>
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Table 1. Terms for degrees of hypothermia by body temperature.

2. Systemic hypothermia

The majority of studies on the induction of TH in acute ischemic stroke therapy have applied whole-body cooling. Therapeutic cerebral hypothermia can be most easily established by either surface cooling or systemic endovascular infusion cold saline [11, 12]. In clinical stroke cases, surface and endovascular cooling have both been used for successful whole-body hypothermia induction and maintenance.
The sentinel study on therapeutic hypothermia exclusively considered “surface cooling,” cooling with ice packs or air-circulating cooling blankets/mattresses. This study demonstrated improved survival outcomes in cardiac arrest patients with therapeutic hypothermia [5, 12]. The Cooling for Acute Ischemic Brain Damage (COOL AID) study additionally showed that moderate therapeutic hypothermia (target temperature 32°C by surface cooling) in patients with acute ischemic stroke is feasible and can be accomplished safely by surface cooling [13]. Surface-cooling methods are easy to use and permit early treatment initiation, which makes them an attractive option. However, there are numerous logistical problems associated with surface cooling that outweigh its benefit.

Systemic endovascular infusion methods reduce body temperature invasively using intravenously placed cooling catheters or intravenous cold infusions of isotonic saline into a major systemic blood vessel. The safety of endovascular cooling in patients with acute ischemic stroke was assessed in both the COOL AID II study [14] and the Intravascular Cooling in the Treatment of Stroke (ICTuS) study [15]. The approach was shown in both cases to reduce body temperature more rapidly than surface cooling could accomplish, and since a temperature probe is embedded in the catheter, precise temperature monitoring and regulation was far superior to surface-cooling methods. The disadvantages of systemic endovascular hypothermia induction stem from its invasive nature; the method carries a much higher risk of deep venous thrombosis (DVT), bacteremia, and sepsis than surface cooling [16, 17]. Additionally, the Intravascular Cooling in the Treatment of Stroke-Longer tPA Window (ICTuS-L) study results showed a statistically significant increase in the occurrence of pneumonias in patients receiving systemic endovascular TH [18].

Unfortunately, whole-body cooling by either method creates a number of serious complications. Chiefly, whole-body cooling frequently causes shivering and dermal vasoconstriction, which can complicate effective progression to optimal cooling ranges; whole-body cooling frequently requires 3–7 h to reach target temperatures [19]. Shivering also raises intracranial pressure (ICP) and requires the use of several pharmacological agents to inhibit these effects along with skin warming to address physical discomfort [20, 21]. Another side effect of whole-body cooling is the risk of shear-induced platelet aggregation, which can develop as blood viscosity increases at low temperatures [22]. Even a minor amount of coagulation can cause a blockage of the microcirculation of the brain and heart, which ironically creates the exact problem that hypothermia attempts to treat [21]. Furthermore, whole-body cooling increases the likelihood of ventricular fibrillations, bradycardia, reduced cardiac output, hemostatic or hemorrhagic changes, decreased urine output, and metabolic dysfunction [14, 23, 24]. With this extensive list of severe complications, a more graceful therapeutic modality is urgently needed.

3. Hypothermia via local endovascular infusion

Recently, the selective induction of hypothermia into the ischemic region using an endovascular microcatheter has garnered attention as a novel strategy to optimize the neuroprotective benefits of therapeutic hypothermia with the myriad of comorbidities accompanying
full-body cooling. In contrast to other cooling methods, which require hypothermia to slowly spread into the ischemic region, local endovascular infusion (LEVI) reduces infarct temperatures effectively by perfusing ice-cold saline directly to the ischemic region. This allows for more rapid achievement of target temperatures and permits greater specificity of hypothermia while avoiding the side effects of systemic cooling. During these procedures, an infusion microcatheter, guided to the site of the lesion via the guide catheter over a microguidewire, is advanced distally to the site of occlusion, and cold saline is perfused [25] for a variable length of time, usually from 5 to 30 min. The logistics of actually performing LEVI in humans are relatively simple, as this is a normal part of performing endovascular interventions for many neuroendovascular surgeons [26]. Therefore, it is expected that this new therapy could easily be added to an angiography suite [27].

LEVI has been tested in animal models of stroke both before and after reperfusion. Pre-reperfusion flushing was first proposed by Ding et al. [28], when the technique was used in a transient middle cerebral artery occlusion (MCAO) rat model. The study produced a 65% reduction in infarct volumes and 61% reduction in leukocyte infiltration when resolution of a 2-h middle cerebral artery occlusion was preceded by LEVI (23°C saline infused at 2 mL/min for 3–4 min) [28]. Pre-reperfusion LEVI has since been shown to reduce infarct volumes by 75 [29] to 90% [30] and significantly conserve motor function both hours and weeks after stroke [29, 30]. Post-reperfusion LEVI has also been considered in some studies, in which a catheter was introduced into the internal carotid artery after blood flow to the ischemic territory had been reestablished [31, 32]. Significant improvements in both infarct volume and functional recovery were observed in every post-reperfusion LEVI trial tested, but these improvements were not as pronounced as those from pre-reperfusion LEVI.

Although the majority of current experimental data on LEVI in stroke are based on rat models, a few large animal studies that have been conducted are equally encouraging. A recent investigation using swine showed that LEVI significantly reduced infarct volumes following 4–4.5-h MCAO (the longest delay of hypothermia in any LEVI large animal study) [33]. The credibility [34], safety, and efficacy of LEVI in Rhesus monkeys were also confirmed, as infusion of cold-lactated Ringer’s solution was used to achieve statistically significant degrees of peri-infarct cooling without apparent vasogenic edema or other comorbidities [35]. Additionally, the safety and feasibility of LEVI was recently verified in humans [36]. In nine human patients with partially or completely treated cerebrovascular diseases undergoing diagnostic cerebral angiogram, 7°C LEVI at ~33 mL/min for 10–13 min was able to reduce jugular venous blood temperature (a proxy for brain temperature) by 0.84°C while reducing rectal temperature by 0.15°C and having no significant effects on vital signs. LEVI was also recently implemented in patients actively undergoing ischemic stroke (within 8 h of symptom onset), which confirmed the safety and feasibility of the procedure [25]. The neuroprotective efficacy of LEVI, however, remains to be established in a clinical setting.

Despite recent milestones in LEVI testing, several systematic obstacles have hindered widespread acceptance. Chief among these obstacles is heterogeneity of experimental designs. Since TH is only widely used for cardiac arrest, the majority of studies utilize a global ischemia model, which has been found to unfaithfully simulate the physiological conditions of
focal ischemia [37]. Hypothermia-based investigations also vary in animal model, animal age, duration of ischemia, duration of hypothermia, depth of hypothermia, method of hypothermia induction, and rate of cooling, all of which have consistently been shown to play critical roles in the efficacy of TH treatments. Additionally, current animal models have failed to adequately simulate the reaction of a human to such an intervention. While the majority of LEVI studies have used rat models, rats have been widely criticized for their poor translatability to clinical practice [38]. There even exists heterogeneity among the species; rats of similar strains from different suppliers have been found to show variations in response to ischemia [12]. Given that stroke accounts for 9% of deaths worldwide and ~25% of stroke survivors are permanently disabled [39], such a promising therapy is in serious need of further exploration.

3.1. Benefits of LEVI over systemic infusion

LEVI is an optimized version of general TH. As such, its mechanisms of neuroprotection are predominately the same as those of full-body cooling. However, LEVI retains a few unique features that make it considerably more effective than global cooling. These features are summarized in the present section.

3.1.1. Maximized rate of cooling

Although there is no consensus on the exact treatment window for therapeutic hypothermia, it would be difficult to dispute the time-sensitive nature of hypothermia induction [31, 40]. While one author found that TH is ineffective after 45 min of ischemia [41] and most others have found neuroprotective efficacy when induction follows 2–3 h of ischemia [30, 42], there is a strong consensus that this efficacy diminishes over the course of hours. Considering that surface-cooling methods frequently take 3–7 h to reach target temperatures [19], it would be impossible for any stroke patient to fall within an optimal treatment window. By contrast, LEVI can establish target temperatures in a matter of minutes [36]; in a 300-g localized cerebral infarct, LEVI attained target temperatures 30 times faster than classic surface cooling and 10–20 times faster than systemic infusion of cold saline into the inferior vena cava [27]. The time saved by using LEVI translates to superior degrees of neuroprotection and an improved quality of life for ischemic stroke patients.

3.1.2. Metabolite washout and attenuated hyper- or hypoperfusion

One mechanism by which ischemic stroke damages the brain is through postischemic hyperperfusion. Under ischemic conditions, brain cells are forced to conduct anaerobic respiration, the byproducts of which (lactate, prostaglandins, and carbon dioxide) are vasodilatory at elevated levels [43]. In the absence of adequate perfusion, these vasodilatory metabolites accumulate in the ischemic region and trigger an excessive vasodilation once perfusion is restored. Literature on postischemic hyperperfusion has been discrepant, but suggests that the phenomenon is associated with larger infarcts and early death [44, 45]. This “luxury reperfusion” has been implicated in post-reperfusion edema formation, the primary cause of death within 1 month of ischemic stroke [45, 46]. While hypothermia prevents intracranial-pressure elevations by itself, LEVI provides an additional protective mechanism by washing out
vasodilatory metabolites built up during the ischemic period, which minimizes the extent of hyperperfusion-related injury [29, 47]. As evidence of this mechanism, fast warm (37°C)-saline LEVI has been shown to significantly reduce infarct volumes and improve functional recoveries compared to systemic infusion of warm saline [28].

Pre-reperfusion flushing also significantly reduces leukocyte infiltration and ICAM-1 expression in the peri-infarct vasculature [28, 48], leading to improved posts ischemic perfusion. Luan et al. showed that LEVI was able to reduce cerebral poststroke ICAM-1 expression and leukocyte infiltration to a significantly greater degree than that of local warm-saline infusion or systemic cold-saline infusion were able to [48]. Other studies have reported similar reductions in ICAM-1 expression and infiltration/activation of PMN leukocytes and microglia [49]. These data imply that the neuroprotective advantages of LEVI over systemic infusion rely partially on its metabolite-washout ability and subsequent improved perfusion.

3.1.3. Drug delivery into ischemic territory through LEVI

In addition to the hypothermia-associated benefits of cold-saline infusion, LEVI allows for coadministration of neuroprotective drugs directly into the ischemic region along with hypothermic fluids, which maximizes local drug concentrations while minimizing systemic drug concentrations, thereby circumventing dose-dependent systemic side effects [50]. Preliminary studies using LEVI with neuroprotective drugs have shown exceptional promise; a 2012 study by Song et al. found that LEVI of a magnesium sulfate solution at 15°C caused a 65% reduction in infarct volumes compared to a 48% reduction from LEVI alone [51]. Similar results were found following LEVI of a 20% human albumin solution cooled to 0°C [52]. Normothermic local infusion of drugs has shown potential as well, as LEVI of erythropoietin at room temperature reduced infarct volumes by 21% (significant compared to control), decreased apoptosis in the ischemic core and penumbra, and significantly preserved neurological scores [53].

LEVI can also aid in drug permeation into the brain parenchyma. Blood-brain barrier (BBB) impermeability has been described as the most important factor limiting the growth of neurotherapeutic drugs [54] and remains a challenging issue today. However, BBB breakdown is a natural product of cerebral ischemia, which allows for the perfusion of drugs into the brain parenchyma that would otherwise be prevented from reaching their target [50]. When coupled with LEVI-based drug administration, BBB breakdown can be capitalized on to provide benefits for stroke therapy. This hypothesis was confirmed experimentally in a 2007 study by Woitzik et al. in which microcatheter-based infusion of MK-801 (an NMDA receptor antagonist) into the ischemic region resulted in 30% smaller infarct volumes at 24 h after infusion than when MK-801 was infused systemically [50]. MK-801 has shown significant neuroprotective potential, but has not attained clinical acceptance due to significant side effects when administered at high enough doses to be effective when infused systemically [55], a problem nullified by LEVI-based administration. While LEVI with neuroprotective drugs has never been tested in a clinical setting, it is possible that the combination could open the door for the use of neuroprotective pharmacotherapies that would otherwise be prohibited from reaching target tissues [50, 56].
4. Mechanisms underlying hypothermia-induced neuroprotection

In addition to LEVI-specific neuroprotective mechanisms, LEVI benefits from neuroprotective mechanisms of therapeutic hypothermia in general. These mechanisms exhibit significant redundancy, as they affect multiple steps in several parallel pathways of hypoxia-induced brain injury. Hypothermia primarily exerts its neuroprotective effects by slowing essential metabolic processes while preserving life, which subsequently attenuates pathways involved in excitotoxicity, free radical production, inflammation, edema, and apoptosis [12, 37, 57, 58]. However, a common theme in literature on the topic is consensus on effects and uncertainty of mechanisms. While virtually every paper finds TH administration to be neuroprotective, there is very little agreement on how this works. This is due, in part, to the correlative goal of most studies. The majority of work on the topic identifies alterations in the levels of one indicator or another when TH is implemented, but fails to elucidate exactly where TH exerts its neuroprotective effects. While this is valuable information, without a causative component, these studies always leave the door open for the participation of a third variable. In light of frequently conflicting findings, this section features few concrete lessons from the literature. Rather, we attempt to discuss the pathways that TH acts on, and consider the most likely points at which TH exerts its neuroprotective effects.

4.1. Metabolic crisis

The primary culprit of ischemia-induced brain damage is oxygen-supply cessation, which initiates a cascade of secondary problems. In the absence of oxygen, neurons are unable to generate high-energy metabolites, which prohibit effective maintenance of ion gradients. Ion-gradient breakdown leads to involuntary depolarization, which allows for excessive glutamate release. This wave of glutamate then stimulates NMDA and AMPA receptors, which results in increased intracellular calcium levels and ultimately leads to excitotoxicity, a phenomenon characterized by mitochondrial membrane depolarization, caspase activation, production of reactive oxygen and nitrogen species, and apoptosis [37, 59]. In addition to excitotoxicity, ion-gradient breakdown causes Na\(^+\) to build up in brain cells and in particular astrocytes. This establishes an osmotic gradient favoring the movement of water into astrocytes (and to a lesser extent, all other brain cells), thereby creating cytotoxic edema [60]. The edema increases intracranial pressure and ultimately exacerbates brain damage (Figure 1).

Hypothermia combats this cascade at several points (Figure 1). Reduced brain temperatures have been shown to lower cerebral metabolic rate by 5% for every 1°C reduction in body temperature, allowing for prolonged maintenance of ion gradients (preventing excitotoxicity) and minimized need to conduct anaerobic respiration, thereby diminishing the extent of reperfusion injury [58]. In patients with traumatic brain injury who received therapeutic hypothermia to 32–33°C, cerebral oxygen consumption was reduced to 27% after 24 h of hypothermia [61]. Hypothermia has also been shown to reduce the production of glycolytic intermediates by an average of 30% and tricarboxylic acid (TCA) cycle intermediates by 30–70% [62]. Alternatively, ratios of phosphocreatinine:inorganic phosphate and adenosine triphosphate (ATP):inorganic phosphate seem to increase slightly during
transient hypothermia, implying that the real energy conservation mechanism at play is one of the slowing energy-consuming reactions, rather than slowing glycolytic flux [62]. Hypothermia has also been routinely reported to improve ATP recovery after reperfusion [37]; mild hypothermia has led to a 10–20% increase in the rate of metabolic recovery in the first 10–25 min after reperfusion compared to normothermic animals [63], a finding echoed in other studies [62, 64]. It is possible, then, that the primary energy conservation mechanism that underlies TH is that of accelerated energy recovery after reperfusion rather than energy preservation during hypoxia. However, while metabolic depression during hypothermia has been well documented as a phenomenon, its underlying mechanism is still poorly understood. Thus, the points at which hypothermia exerts its neuroprotective effects are unclear, and whether its main mechanism of neuroprotection involves cellular respiration at all remains to be elucidated.

Hypothermia has also been shown to prevent anoxic depolarization. In an aged rat model, mild hypothermia was shown to completely inhibit the efflux of excitatory amino acids (glutamate and aspartate) while significantly increasing the release of the inhibitory amino acid taurine [65]. While no mechanism has been firmly tied to this phenomenon, several have

Figure 1. The figure describes the pathogenesis of stroke as it relates to ischemia-induced metabolic crisis. The points at which hypothermia exerts its neuroprotective effects remain largely unclear. Studies have shown that TH attenuates a multitude of steps in the cascade of ischemia-induced brain damage compared to stroke without hypothermia, but whether the observed attenuations are direct effects of TH or byproducts of upstream attenuations has yet to be elucidated. As such, blue font indicates steps discussed in the present review that hypothermia has been shown to attenuate. Black font indicates steps that we have not discussed in the present review, but does not necessarily indicate that these steps are unaffected by hypothermia.
been speculated. TH has been reported to prevent activation of protein kinase C (PKC) and calcium-calmodulin kinase II during ischemia, both of which are associated with neurotransmitter release [65]. Therapeutic cooling also attenuates ischemia-induced downregulation of the GluR2 (glutamate receptor 2) CA1 subunit, which is responsible for limiting Ca$^{2+}$ influx through AMPA receptors in a global cerebral ischemia model [66]. It is also possible that this facet of hypothermic neuroprotection is accomplished by the preservation of ion gradients due to metabolic downregulation. However, some reports have suggested that hypothermia simply delays anoxic depolarization rather than preventing it [67]. In light of conflicting research on the topic, it is likely that multiple mechanisms are at play, culminating in the robust excitation prevention associated with therapeutic hypothermia.

Hypothermia has also been found to combat cytotoxic edema after ischemic stroke (Figure 1). This edema is largely mediated by aquaporin 4 (AQP4), which is expressed in the glial-limiting membranes, ependyma, and pericapillary foot processes of astrocytes [68]. In mice, AQP4 knockout has been associated with reduced infarct sizes, decreased brain water content, and improved neurological and survival outcomes [60]. While the brain naturally downregulates AQP4 expression following hypoxia [60], hypothermia has been shown to augment this downregulation [60, 69, 70]. It is possible that this downregulation is a downstream effect of TH. In experimental models, AQP4 levels in astrocyte cell membranes were increased by increased lactic acid concentration, but AQP4 mRNA levels were unchanged, which implies that the observed increases in membrane-bound AQP4 came as the result of redistribution or posttranslational modification, rather than increased expression [71]. Several other mechanisms have also been proposed for this upregulation [72]; thus, the specifics of ischemia-induced aquaporin modulation have still yet to be fully elucidated.

At the molecular level, several studies have implicated immediate induction of early gene expression (miRNAs) and cellular stress response (heat-shock proteins, HSPs) activation in hypothermia-induced neuroprotection. Hypothermia has been shown to suppress transcription of some pro-inflammatory molecules (interleukin (IL)-1β and osteopontin) and enhance transcription of anti-inflammatory substances (HSP70) [73]. The duration of postreperfusion hypothermia seems to play a role in the modulation of transcriptional rate, as the expression of numerous genes differs when hypothermia is sustained for 8 h compared to 4 h. One such gene is early growth response-1 (Egr-1), which is an early regulator of other pro-inflammatory mediators (IL-1β MCK-1, and MIP-2) [73]. This is consistent with other reports on the topic, which suggests that Egr-1 is the key component modulated by TH. However, information regarding early cellular response to ischemia and hypothermia has largely been conflicting, leaving the specifics of its involvement uncertain [38] and inconsistent [31, 32].

While there exists a general consensus that TH is neuroprotective, the precise mechanisms of this effect are still very much theoretical. Additionally, if TH attenuated metabolic crisis alone, it would not be able to accomplish such a robust degree of neuroprotection [74]. It comes as no surprise, then, that suspended animation is just the appetizer in the multicourse meal that is TH-mediated neuroprotection.
4.2. Inflammation and blood-brain barrier breakdown

In stroke therapy, the restoration of blood flow is of chief concern. Surprisingly, however, recanalization is not exclusively beneficial. Reperfusion often initiates a detrimental cascade, collectively termed ischemia/reperfusion injury, which can be disastrous; in some animal models, reperfusion after an extended period of ischemia caused larger infarct volumes than if the occlusion had been left permanently [45]. Reperfusion injury is a complex, multifaceted injury cascade initiated by sterile inflammation from anoxic tissue damage, and propagated by both the innate and adaptive immune systems and complement system [75, 76].

Mechanistically, ischemia/reperfusion injury is initiated by the aberrant Ca\(^{2+}\) influx characteristic of ischemic stroke, which activates phospholipases and eventually results in the production of pro-inflammatory mediators from microglia, including proteases, leukotrienes, IL-1\(\beta\) IL-6, NO, and tumor necrosis factor (TNF)-\(\alpha\) [77]. These mediators contribute to post-reperfusion insult directly, by increasing vascular permeability, and indirectly, by increasing endothelial ICAM-1 expression and serving as potent chemotactic agents for polymorphonuclear leukocytes, both of which increase leukocyte extravasation into the brain parenchyma [46, 78]. The pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) is likely the cause of this upregulation, as it is responsible for inducing the expression of IL-1\(\beta\) IL-6, TNF-\(\alpha\), and ICAM-1 [78]. In addition to recruiting leukocytes to the infarct site, IL-1\(\beta\) and TNF-\(\alpha\) have also been found to increase the production of matrix metalloproteinases (MMPs) [79]. MMP-2 and MMP-9 have been shown to contribute to vasogenic edema by degrading extracellular matrix components during ischemic stroke, and MMP-9 knockout mice experience reduced infarct volumes and less severe motor deficits than wild-type mice [79]. The effect of MMPs ultimately perpetuates the development of inflammation and edema, which further encourages leukocyte extravasation. Once leukocytes enter the brain tissue, they produce ROS and pro-inflammatory factors of their own, thereby creating a viscous cycle of brain injury, inflammation, and blood-brain barrier (BBB) breakdown (Figure 2).

A common effect of reperfusion injury mechanisms is BBB disruption. Reperfusion activates matrix-degrading proteases within hours, which makes the vessels particularly leaky and allows for migration of albumin and other blood proteins into the brain parenchyma within 4–6 h of BBB disruption [72]. Water osmotically follows these proteins, thereby creating vasogenic edema, which may increase brain water content by more than 100% in poorly perfused regions [72, 80]. Vasogenic edema is the primary cause of death within the first month of an ischemic stroke [46], as it increases intracranial pressure (ICP) and compresses cerebrovascularity within the inflexible confines of the skull, causing further ischemia and eventually brain herniation [56].

Therapeutic hypothermia is able to confer anti-inflammatory neuroprotection by reducing the secretion of pro-inflammatory cytokines (IL-1\(\beta\) TNF-\(\alpha\), and IL-6) and inflammatory mediators (reactive oxygen and nitrogen species, E-selectin, and HMGB1) [81]. TH can also prevent leukocyte extravasation into neural tissue directly by reducing the endothelial expression of ICAM-1 [27, 48]. ICAM-1 is constitutively expressed by endothelial cells at very low levels, but the expression is precipitously increased following endothelial damage when it functions as an attachment point for the CD11/CD18 integrin of leukocytes (preceding extravasation
ICAM-1 knockout mice are resistant to cerebral ischemic injury [83], and antagonization of CD11/CD18 has been shown to substantially reduce leukocyte infiltration and subsequent cerebral edema (Figure 2) [82]. These effects seem to be associated with the inhibition of the extracellular signal-regulated kinase (ERK) pathway (Figure 2). ERK plays a significant role in the regulation of cell survival signals, and in the brain it is involved in responses to stress stimuli, including glutamate...
receptor stimulation and oxidative stress [84, 85]. ERK has been shown to contribute to NO and TNF-α secretion, and inhibition of the pathway prevents the release of excitotoxic amino acids following focal ischemia [86]. Transient hypothermia has been shown to reduce microglial activation, which translated to reduced phosphorylation (activation) of ERK and decreased IL-6 and TNF-α secretion [84]. However, induction of hypothermia in conjunction with U0216 (an ERK inhibitor) provided equal functional recovery to rats that did not receive U0216, implying that poststroke functional recovery progresses independently of ERK signaling [87]. Hypothermia has also been shown to reduce ICAM-1 expression in microglia in correlation with the ERK pathway, as administration of TH led to decreases in the activation of ERK as well as the inhibition of ICAM-1 expression [84].

Hypothermia-associated decreases in the expression of ICAM-1, IL-1β and TNF-α may also be due to attenuation of the NF-κB pathway (Figure 2). Therapeutic hypothermia has been shown to increase the expression of HSP70 in ischemic brains (but not in non-ischemic brains), and reports have suggested that HSP70 stabilizes NF-κB, thereby preventing its phosphorylation (activation) [73]. However, other NF-κB-associated proteins contribute as well. This pathway is puzzling, as the mechanism of NF-κB suppression varies depending on the type of ischemia. In models of focal ischemia, hypothermia suppresses NF-κB activity by inhibiting the activity of NF-κB kinase (IKK), a protein essential for degradation of the NF-κB inhibitor (IκB). In models of global ischemia, nuclear NF-κB levels in hypothermic subjects were still below normothermic levels, but IKK and IκB levels were unchanged [78]. These results are surprising, but emphasize the complexity of stroke pathogenesis and TH-associated neuroprotection. Moreover, regardless of the precise mechanism, therapeutic hypothermia seems to serve a beneficial role in NF-κB-associated neuroprotection.

Hypothermia can also prevent BBB breakdown directly. Nagel et al. recently found that TH increased functional recovery and reduced MMP-2 and -9 activities to the same degree as normothermic application of the MMP inhibitor minocycline, and that the application of TH in conjunction with minocycline was only marginally more effective than either by itself [88]. In addition to decreasing MMP activity, minocycline has been shown to decrease MMP production at the transcriptional level, and this report suggested that TH functions in the same way [88]. Other groups have found similar results, and this TH-induced MMP downregulation indeed translated to smaller infarct volumes and improved functional recovery [79, 89]. These data consistently show that TH is a powerful downregulator of MMP expression and activity, and that the modulation of MMP function leads to marked improvements in big-picture end goals of stroke therapy (decreased infarct volume, increased functional recovery, etc.) (Figure 2).

4.3. Apoptosis

Following the initial ischemia-induced insults (hours to days), long-term brain damage (days to weeks) is greatly influenced by cellular proapoptotic mechanisms. Hypothermia has been shown to affect several aspects of apoptotic cell death in both the intrinsic (intracellular-mediated) and extrinsic (receptor-mediated) cell death pathways, and ultimately prevent apoptosis after experimental stroke (Figure 3) [37].
The extrinsic apoptotic pathway is initiated by ligand binding to cell death receptors; the best studied being the FAS-ligand (FASL) and its receptor, FAS. When FASL interacts with FAS, it triggers the intercellular assembly of death-induced-signaling complexes (DISCs), which leads to caspase 8 activation. Activated caspase 8 then triggers a caspase activation cascade resulting in the stimulation of apoptosis-inducing proteins such as caspase 3, thereby mediating cell death (Figure 3).

Hypothermia affects this pathway at multiple levels (Figure 3). Cooling has been shown to suppress the expression of caspase 8, caspase 3, FAS, and FASL [90]. Additionally, there is evidence that the FAS-FASL complex must be cleaved from the cell membrane by MMPs before becoming active [91]. TH has been shown to reduce levels of both MMPs and soluble...
FASL in cooled rat brains [91], so is possible that the reduction in levels of these downstream effectors is simply the byproduct of inhibiting the FAS-FASL cleavage. While there are little available data to this end, the fact remains that, by one mechanism or another, hypothermia significantly reduces the production of a number of extrinsic apoptotic pathway intermediates, which translates to the preservation of penumbral tissue.

The intrinsic apoptotic pathway is triggered by intracellular cell stress signals including hypoxia, DNA damage, and cellular detachment from the extracellular matrix. These signals initiate apoptosis by disrupting the balance between proapoptotic Bcl-2 family members (BID, BAX, BAD, etc.) and anti-apoptotic Bcl-2 members (Bcl-2, Bcl-x, etc.) by a variety of mechanisms. Bcl-2 and Bcl-xL have both been found to be upregulated in neurons surviving hypoxia, while proapoptotic Bcl-2 members are highly expressed in neurons that will eventually die from hypoxic damage [37]. The imbalance between pro- and anti-apoptotic Bcl-2 members leads to the liberation of cytochrome C from the mitochondrial intermembrane space into the cytosol where it couples with APAF1 to form an apoptosome. The apoptosome activates caspase 9, which triggers a caspase activation cascade resulting in the activation of caspase 3 and apoptosis (Figure 3).

Hypothermia exerts its neuroprotective effects at several points along the intrinsic apoptotic pathway (Figure 3). TH has been found to inhibit BAX overexpression 4 h after 30 min of partial ischemia while having no effect on Bcl-2 expression [92]. TH has also been shown to diminish cytochrome C release without modifying BAX or Bcl-2 expression. This study did not observe caspase activity, which implied that TH endowed neuroprotection functions independently of caspases [93]. Interestingly, the same group found that hypothermia increased Bcl-2 expression in a global ischemia model [94], which underlines the importance of designing studies specific to local cooling in focal ischemia models. Additionally, mild hypothermia has been found to decrease cytochrome C translocation 5 h after reperfusion while leaving levels of caspase 9 and caspase 3 unchanged [74]. The conflicting nature of these findings leaves the point at which TH exerts its protective effects in question, but emphasizes the intricacy of TH-mediated neuroprotection.

Some of the anti-apoptotic effects of TH are mediated through the anti-apoptotic factor Akt/protein kinase B (Figure 3). Hypothermia attenuates decreases in Akt dephosphorylation (inactivation) after hypoxia [90]. In response to growth factors including BDNF (brain-derived neurotropic factor), membrane receptor tyrosine kinases activate PI3 kinase, which activates Akt via phosphorylation (p-Akt), thereby allowing it to phosphorylate (inhibit) numerous proapoptotic factors, including BAD, caspase 9, and forkhead transcription factor (FKHR) [90, 95]. Under normal physiological conditions, these proteins are phosphorylated by Akt, and their dephosphorylation can have severe repercussions. Dephosphorylation of BAD allows it to migrate into the mitochondria where it triggers the release of cytochrome C [91]. Dephosphorylated FKHR functions as a transcription factor to encourage overexpression of FASL and BIM [90]. Activation of caspase 9 activates a caspase cascade that results in apoptosis.

In normothermia, poststroke p-Akt levels fluctuate constantly; Zhao et al. found that, in normothermic rats, p-Akt levels decreased 30 min after stroke, increased at 1.5 and 5h, decreased
at 9 and 24h, and increased again at 48h. Moderate hypothermia was found to stabilize these fluctuations at every time point except 24h, which translated to reduced infarct volumes and improved functional recovery up to 2 months after hypoxia. Interestingly, the reduction in infarct volumes was considerably less pronounced when TH was administered in conjunction with the PI3K inhibitor LY294002, although infarcts were still substantially smaller than in control animals [90]. It is very likely that this pathway provides a significant portion of TH-mediated neuroprotection. In line with this premise, mild hypothermia has also been found to inhibit the expression of caspase-3 and Fas after resolution of focal ischemia, which also translated to significantly decreased infarct volumes [96]. Hypothermia has also been shown to augment BDNF expression during cerebral ischemia [97], as well as attenuate the decrease in the Akt activity after stroke [90], so it is also possible that the effects of hypothermia on Akt activity are mediated at the level of BDNF.

While direct enhancement of the Akt pathway likely constitutes a portion of TH-mediated neuroprotection, cerebral cooling intervenes at other steps in the pathway as well. The PI3K/Akt pathway is inhibited by phosphatase and tensin homologue (PTEN), which de-phosphorylates upstream activators of Akt. PTEN is inhibited by phosphorylation (p-PTEN), and p-PTEN levels seem to play a crucial role in TH-mediated neuroprotection. Hypothermia has been found to stabilize p-PTEN levels more effectively than levels of p-Akt and other PI3K/Akt pathway participants (p-PDK1, p-GSK3β p-FKHR) [37]. A recent investigation from Lee et al. found that TH administered 15 min before reperfusion led to massive decreases in infarct volume while TH administered 15 min after reperfusion only had modest infarct reductions. Interestingly, while early and late TH had nearly identical effects on levels of p-Akt and other proteins, only early TH maintained high levels of p-PTEN [98]. Additionally, independent of hypothermia, PTEN inhibition was recently shown to confer a 75% reduction in infarct volume in rat models [95]. PTEN clearly plays a critical part in the story of neuroprotection, and should not be neglected in future investigations on the topic.

4.4. Long-term neuroprotection

There is compelling clinical evidence of neuroprotection with prolonged moderate cerebral hypothermia initiated within a few hours after hypoxia-ischemia and continued through the resolution of ischemia in term infants and adults [99–101]. The mechanisms underlying the neuroprotection are currently under investigation. Volser et al. showed that during the post-ischemic phase, the brain naturally activates restorative mechanisms to counteract the effects of the ischemic insult even without the induction of hypothermia [102]. This study, among others put forth the idea of long-term neuroprotection following an ischemic event in the brain. A study by Feng et al. went a step further and found that acute brain insult led to stimulation of neural stem cell proliferation, particularly in the subventricular and hippocampal subgranular zone, corroborating long-term neuroprotection [103]. However, evident from the lasting symptoms of acute ischemia, the brain is unable to completely regenerate and recover from the injury on its own. Thus, there is a dire need for the development of effective regenerative techniques and therapies to maximize patient recovery. This is where LEVI and hypothermia can be used to further the recovery of the brain.
Over the past decade, researchers have proposed the following mechanisms of long-term neuroprotection: neurogenesis, angiogenesis, gliogenesis, preservation of the integrity of neural networks, and inhibition of apoptosis [55]. These mechanisms will be discussed in detail below.

4.4.1. Neurogenesis

Contrary to prior belief, neurogenesis is a common event observed in the brain and while it is primarily limited to two neurogenic areas of the brain, the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, this process plays an important role in maintaining normal brain function [104, 105]. Two potential mechanisms can be attributed to neurogenesis: enhanced differentiation of neuroprogenitor cells into neurons and preferential differentiation of neuroprogenitor cells toward neurogenesis.

The formation of new neural cells from neural progenitor cells has been identified as a major contributor to new populations of neurons, and TH seems to encourage this formation. An *in vivo* study by Silasi et al. found that when forebrain ischemia was induced in adult rodents, mild hypothermia following the ischemic event led to significantly increased neurogenesis in the dentate gyrus when compared to control groups with no hypothermia induction following an ischemic event [106]. Moreover, a very recent study in a neonatal hypoxic-ischemic injury mouse model showed that hypothermia provided partial protection for neural stem and progenitor cells (NSPCs) in the dentate gyrus subgranular zone, which may facilitate the recovery of function after injury and does not impair the proliferation of NSPCs during recovery [107]. This TH-mediated neurogenesis is thought to confer a more robust, long-term conservation of brain function than would be seen in normoxic stroke patients.

Preferential differentiation of neuroprogenitor cells into neurons also plays a major role in neuroprotection. Interestingly, an *in vivo* study found that cooling of rat brains to 33°C under hypoxic conditions led to an inhibition of hypoxia-induced apoptosis of proliferating neural stem cells and an increase in preferential maturation of neural progenitor cells into neural cells in the striatum [108]. Moreover, an *in vitro* study by Saito et al. found that moderate hypothermia to 32°C prevented apoptosis, preserved the naivety of neural stem cells, and led to lower expression of GFAP in neural stem cell culture, indicating less glial differentiation [109].

On the other hand, a study from early 2016 found that in aged rats, hypothermia induced by H$_2$S gas for 24 h after resolution of an MCAO only provided temporary therapeutic benefit and did not correlate with enhanced neurogenesis in the subventricular zone or infarcted area [110]. However, the duration of hypothermia induction in this study was shorter than the duration of hypothermia used in most clinical trials (24–48 h) and thus led to suboptimal hypothermia which is reflected in the temporary therapeutic effects [110]. Additionally, the use of hydrogen sulfide to induce hypothermia may not be representative of the conventional hypothermia-inducing agents used in other animal studies. H$_2$S is a weak and reversible inhibitor of oxidative phosphorylation, thus causing a suspended animation state with hypothermia [111]. It is quite possible that the mechanism of induction of hypothermia by H$_2$S may have interfered with various long-term protective mechanisms observed in other studies and in clinic using conventional hypothermia techniques.
4.4.2. Angiogenesis

Angiogenesis is a normal yet important biological process that is highly regulated and leads to the formation of new blood vessels during development, wound repair, and reproduction [111]. A study on rats by Xie et al. found that mild hypothermia enhanced angiogenesis in focal cerebral ischemia by increasing microvessel diameter, number of vascular branch points, and overall vessel surface area [112]. This was found to be a brain-derived neurotrophic factor (BDNF)-dependent process. Moreover, another study using a rat MCAO model showed that the injection of BDNF fused with a collagen-binding domain (CBD-BDNF) into the lateral ventricle specifically bound to collagen of the ventricular ependyma and consequently led to neural regeneration, angiogenesis, and reduced cell death [113]. This study further confirms the pro-angiogenic activity of BDNF in ischemic conditions. Vascular endothelial growth factor (VEGF) upregulation has also been found to correlate with acute cerebral ischemia [114, 115]. A very recent prospective cohort study observed increased brain perfusion over the first month in term-asphyxiated newborn babies treated with hypothermia during the first few days of life. This increase in brain perfusion came as a result of increased angiogenesis, which was found to be associated with VEGF expression in the injured brain of asphyxiated newborns treated with hypothermia [116]. VEGF has been consistently shown to increase angiogenesis, which translates to increased functional recovery in the months following an ischemic stroke [117–119].

4.4.3. Gliogenesis

While gliogenesis refers to the development of microglia, oligodendrocytes, and astrocytes in the brain, intriguingly, oligodendrocytes have been found to have a similar susceptibility to neurons for cell death. Early studies found that combined deprivation of oxygen and glucose led to selective death of mature oligodendrocytes over other glial cells in vitro [120–122]. In vivo studies have shown that cerebral white matter, specifically oligodendrocytes and astrocytes, are highly vulnerable to focal ischemia [123]. However, in vitro studies have shown that hypothermia increases the number of oligodendrocyte precursors in primary neural and glial cultures from mouse brains and maintains a cell population of oligodendrocyte progenitors in a less well-differentiated state [124]. Recent studies have found that susceptible oligodendrocyte progenitors and mature oligodendrocytes exposed to hypoxia could be protected by deep hypothermia [125]. Another study demonstrated that hypothermia promoted the differentiation and maturation of oligodendrocyte precursor cells (OPCs), and indicated that OPC death was significantly suppressed by hypothermia in vitro, alluding to the fact that hypothermia is protective of oligodendrogiogenesis [126]. More recent studies in fetal sheep have shown that cerebral ischemia is associated with significant loss in total numbers of oligodendrocytes, decreased myelin basic protein expression, and increased microglial activation [127, 128]. However, another study in fetal sheep countered these results by showing that delayed cerebral hypothermia partially protects white matter after global cerebral ischemia by stimulating oligodendrocyte proliferation, reducing microglial induction, and restoring the amount and pattern of expression of myelin basic protein, once again confirming the neuroprotective role of hypothermia toward oligodendrogenesis [129, 130]. Moreover, researchers have found that hypothermia attenuates demyelination, trauma-induced oligodendrocyte cell death, and
overall circuit dysfunction [131, 132]. While a study in preterm fetal sheep found that TH was correlated with an overall reduction in the hypoxia-induced death of immature oligodendrocytes, hypothermia did not prevent the hypoxia-induced inhibition of oligodendrocyte proliferation in the periventricular white matter zone [133, 134]. Most importantly, a recent study in rats found that hypothermia reduced the extent of hypoxia-ischemia damage in axons and increased oligodendrocyte lineage proliferation, which was reflected in the increase in myelination of axons and decreases apoptosis and pre-oligodendrocyte lineage accumulation [134]. While an ischemic environment has been shown to be detrimental to oligodendrogenesis and oligodendrocyte survival, hypothermia has been shown to rescue these processes in vivo and in vitro, as discussed above.

Since astrocytes are the largest population of cells present in the ischemic core during the subacute to chronic period of stroke, astrogliogenesis is often considered to be therapeutic following insult to the brain [131, 135, 136]. However, we still lack much information and need more investigation in this area. Most of the current literature suggests astrogliogenesis as detrimental to the brain rather than neuroprotective. As we know, activated astrocytes form the glial scar in the brain following insult or injury [112, 137]. This brings about doubt on whether astrogliogenesis is therapeutic and may actually impede the posts ischemic healing process by forming a glial scar that could hinder neurite growth and synaptogenesis, and lead to leakage of proapoptotic factors from astrocyte gap junctions within the glial scar [138]. Moreover, a very recent study found that in mice, hypoxia diminished the protective function of astrocytes and activated them to initiate astrogliosis in the ischemic region [139].

4.4.4. Preservation of the integrity of neural networks

Neural networks are functional units representing the high complexity and processivity of the brain and thus repair and preservation of this circuitry is the key for recovery from brain injury. Some of the key processes involved in neural network maintenance are axonal and neurite growth, synaptogenesis, and maintenance of neuronal architecture. Studies have found that hypothermia of the brain by 17°C enhanced neurite and axonal outgrowth in brain slices [142, 143]. A recent study on spinal cord injury rat models found that regional hypothermia promoted neurite, axonal, and nerve fiber growth to the point that hind limb function was recovered in these rats, which emphasizes the plasticity and extent of recovery via hypothermia that the central nervous system is capable of [144]. However, deep hypothermia (20°C) followed by subsequent rewarming did not change the stability of dendritic spines or the presynaptic boutons in mouse somatosensory cortex [145]. Moreover, a gene profiling study on rat model of traumatic brain injury found that mild hypothermia had significant effects on gene expression for synapse organization and biogenesis; an analysis
of the hippocampal gene expression profiles of these rats found that 133 genes showed statistically significant changes in expression compared to injured rat in normoxic conditions. Of the 133, 57 genes were upregulated and were responsible for synaptic organization and biogenesis [146]. An in vitro study showed that hypothermia to 33°C following in vitro ischemia decreased the neuronal actin polymerization that reduced spine calcium kinetics, disrupted detrimental cell signaling, and protected the neurons against damage [147]. While hypoxic conditions caused changes in F-actin architecture of dendritic spines, hypothermia decreased the actin modifications in dendritic spines preventing the neuronal death [148]. All of these studies support the notion of spine and synaptogenesis preservation by hypothermia treatment.

In a functional study on ischemic gerbils treated with moderate postischemic hypothermia, the untreated (normothermic) groups experienced a 95% reduction in CA1 cells, while cell counts in the TH group were equivalent to that of sham animals. Additionally, postischemic hypothermia preserved the electrophysiological properties of CA1 neurons, which reflects the functional preservation of neural networks [149]. Moreover, mice subjected to ischemia followed by hypothermia treatment showed neuroprotection against ischemia-induced long-term potentiation (LTP) impairment as well as synaptic plasticity [150]. While there are encouraging studies on mechanisms of neural network preservation by hypothermia treatment, further research is needed to better understand how neuronal networks are preserved in the ischemic and penumbra regions in response to hypothermia.

5. Future research directions

Between 1935 and 2010, cancer, heart disease, and stroke have consistently been in the top five causes of death in the United States [151]. While all three are complex, multifaceted diseases, stroke differs from cancer and heart disease in one critical way; a highly effective, easily administered, cost-effective therapy has already been devised. The main factor hindering significant progress on stroke therapy is not a lack of ideas, but rather a lack of research moving hypothermia toward clinical acceptance. Since TH is still predominately discussed in the context of cardiac arrest, the majority of studies on TH feature a global ischemia (cardiac arrest) model, which cannot always be extrapolated to studies on focal cerebral ischemia. Several papers in the present review alone have arrived at a finding using a global ischemia model that is directly opposed by results from a model of focal ischemia or vice versa [37, 93, 94]. In focal ischemia models, there is significant heterogeneity in experimental methods. Studies on TH in focal cerebral ischemia frequently differ in animal model, animal age, duration of ischemia, duration of hypothermia, depth of hypothermia, method of hypothermia induction, and rate of cooling, all of which have consistently been shown to play critical roles in the efficacy of TH treatments. It is also important to note that the vast majority of investigations on neuroprotective efficacy have used transient occlusion models, which produce much more uniform and encouraging results than those using a permanent occlusion model [37]. This is problematic, considering that an estimated 50% of ischemic stroke patients display vessel occlusion 3–4 days after symptom onset, which is considered
a relatively permanent occlusion [152]. This heterogeneity is likely a large source of conflicting findings, and surely prevents investigators from coming to an agreement on TH mechanisms. Another issue with present research is the goal of hypotheses. While there have been innumerable studies on the mechanisms of hypothermia-mediated neuroprotection, these reports are usually correlative rather than causative, which makes it difficult to derive any concrete, widely applicable mechanisms from the literature. This overall lack of research has hindered publicization of the procedure; given that LEVI was only developed in 2002, many groups are simply unaware that such a procedure has been proposed. For instance, a highly cited 2012 review on the topic discussed numerous problems with global cooling, but failed to mention LEVI to any extent despite the fact that the procedure remedies every problem highlighted in the paper [58]. However, as the body of research on LEVI grows, so too will its clinical acceptance.

Overall, the picture of therapeutic hypothermia-mediated neuroprotection is favorable and encouraging. TH consistently decreases infarct volumes and facilitates short- and long-term preservation of function to an unprecedented degree. Although there is little widespread consensus as to how this is accomplished, a review of the literature is scarce with detrimental effects of TH. While many questions remain to be answered before TH can be consistently implemented in humans, such a promising therapy to such a ubiquitously disastrous disease warrants a significant time investment going forward.

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