Published in final edited form as:

Ann N Y Acad Sci. 2013 March ; 1278: 25–32. doi:10.1111/nyas.12087.

Neuroprotection after cerebral ischemia

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Abstract

Cerebral ischemia, a focal or global insufficiency of blood flow to the brain, can arise through multiple mechanisms, including thrombosis and arterial hemorrhage. Ischemia is a major driver of stroke, one of the leading causes of morbidity and mortality worldwide. While the general etiology of cerebral ischemia and stroke has been known for some time, the conditions have only recently been considered treatable. This report describes current research in this field seeking to fully understand the pathomechanisms underlying stroke; to characterize the brain's intrinsic injury, survival, and repair mechanisms; to identify putative drug targets as well as cell-based therapies; and to optimize the delivery of therapeutic agents to the damaged cerebral tissue.

Keywords

stroke; cerebral ischemia; cerebrovascular disease; neurovascular unit; cell therapy; repair; immune response

Background and perspectives

Following interruption of blood flow to the brain in an ischemic event, cells undergo a series of events, such as loss of ion gradients, including failure of the sodium–potassium pump

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Conflicts of interest

The authors have no conflicts of interest.

Meeting participants list

Cornell University, Ithaca, NY (Nozomi Nishimura, Chris Schaffer); Fukuoka Dental College, Fukuoka, Japan (Hiroaki Ooboshi); Hiroshima University, Hiroshima, Japan (Eisuke Dohi, Shigeru Tanaka); Kyushu University, Fukuoka, Japan (Tetsuro Ago, Nozomi Akimoto, Mami Noda); Louisiana State University, New Orleans, LA (Nicolas Bazan, Ludmila Belayev, Alberto E. Musto); Morehouse School of Medicine, Atlanta, GA (Byron Ford, Donghui Li, Shobu Namura, Zhenfeng Xu); National Institutes of Health/ National Institute of Environmental Health Sciences, Research Triangle, NC (David Miller); Okayama University, Okayama, Japan (Koji Abe); Osaka University, Osaka, Japan (Kazuo Kitagawa); Stanford University, Stanford, CA (Raphael Guzman); Tokai University, Kanagawa, Japan (Shunya Takizawa); Tulane University, New Orleans, LA (David Busija, Prasad V.G. Katakam); University of California San Francisco, San Francisco, CA (Yosuke Akamatsu, Masahito Kawabori, Chih Cheng Lee, Jialing Liu, Midori A. Yenari); University of Washington, Seattle, WA (Kyra Becker, Allison Kunze); Washington University, MO (Jeffrey M. Gidday);

(Na⁺-K⁺), which leads to cellular swelling and cytotoxic edema. As cells switch from aerobic to anaerobic metabolism, metabolic acidosis ensues. Loss of ion gradients also leads to accumulation of intracellular calcium and excitatory amino acid (EAA) release, with activation of corresponding EAA receptors, leading to further calcium influx, mitochondrial dysfunction, and cell death through both necrotic and apoptotic pathways. Upon reperfusion, injured cells elicit a stress response, characterized by upregulation of immediate early and other stress response genes, which, in turn, leads to *in situ* production and/or upregulation of immune modulators, such as cytokines, and to trafficking of circulating immune cells into the ischemic brain. Necrotic cells may also release nucleic acids and other molecules that can act as damage-associated molecular patterns (DAMPs) on immune cells, including microglia, leading to immune cell activation and proinflammatory signaling. Proinflammatory molecules can then activate other proteins, such as matrix metalloproteinases (MMP), involved in the disruption of the blood–brain barrier (BBB) and the extracellular matrix. This worsens ischemic injury by causing vasogenic edema and hemorrhage. Reperfusion and immune cell signaling can also lead to astrocyte activation, with elaboration of prosurvival factors, setting the stage for reparative processes, such as neurogenesis and angiogenesis, as well as gliosis. The initial stress response also leads to induction of various survival factors, such as Akt and the cAMP response element–binding protein (CREB).

The Trans-Pacific Workshop on Stroke was held at Wyndham Riverfront Hotel in New Orleans, Louisiana, on October 17–18, 2012. The workshop was organized by Midori A. Yenari and Hiroaki Ooboshi, along with Shobu Namura and Jialing Liu. The workshop was sponsored by the US-Japan Brain Research Cooperative Program and the Japan-US Science and Technology Cooperation Program Brain Research Division.

Three goals were proposed to develop strong collaborations among investigators in both nations: (1) to exchange increasing knowledge of ischemic stroke, from the basic to clinic level, among researchers in the US and Japan; (2) to identify barriers and gaps that inhibit our complete understanding of the patho-mechanisms underlying stroke; and (3) to identify research areas that should be studied in the future. The scientific sessions were focused on several areas of investigation (Figure 1): injury, survival, and repair mechanisms; potential drug targets and cell-based therapies; delivery of therapeutic agents, such as manipulating the blood-brain barrier; and state-of-the-art imaging of neurovascular changes and for tracking delivered therapeutic agents. Speakers were selected from both countries to cover these areas. Participation of students and junior investigators was encouraged, with an emphasis of diversifying the future workforce of stroke research, particularly in the US. A total of 30 attendees were invited and presented their studies. A schematic of areas discussed as they relate to cell-cell interactions, signaling, and therapeutics within the neurovascular unit is shown in Figure 2.

Neuroprotection and clinical studies against ischemic stroke

The scientific session was started by Kazuo Kitagawa (Osaka University), who has led the field of ischemic tolerance for more than two decades. He presented his recent progress in understanding the molecular mechanism underlying this phenomenon.¹ In the cytoplasm of neurons under non-stimulated conditions, SIK2 is highly expressed, which phosphorylates/sequesters CRTC1. After ischemic stimuli, CaMK isoforms I/IV phosphorylate SIK2, resulting in degradation of SIK2. Consequently, CRTC1 is dephosphorylated, and translocates into the nucleus, binding to the promoter region of CREB. By doing so, this signaling pathway activates CREB-mediated survival genes, such as of BDNF, PGC-1 α , and Bcl2. Interestingly, this pathway (CaMK-SIK2-CRTC1-CREB) seems to be downstream specifically to synaptic NMDA receptor (NR2A-subunit containing) activation

but not to other glutamate receptors. The concept was supported by in vivo findings in SIK2 null mice that showed strong resistance to ischemic stroke. Given the robustness of neuroprotection provided by this signaling pathway, a next question will be how this survival pathway can be triggered pharmacologically, ideally after the onset of stroke.

Another neuronal survival mechanism was proposed by Shigeru Tanaka (Hiroshima University). Gene transfection of G-protein coupled receptor (GPR)-3 to cultured neurons rendered resistance against hypoxia. In contrast, GPR3 gene knockdown by siRNA transfection augmented hypoxic neuronal apoptosis. Consistent with these findings, GRP3 knockout mice are more vulnerable to transient focal cerebral ischemia compared with wild-type animals. GRP3 ligands may have a therapeutic value although it is unknown how the GRP3 activation renders neuronal survival.

The role of autophagy, a cellular mechanism of clearing unnecessary cellular debris, was discussed by Eisuke Dohi (Hiroshima University). He showed that disrupting chaperone-mediated autophagy worsened neuron death due to hypoxia.

The neuroprotective efficacies of docosahexaenoic acid (DHA), a fish oil component, and its derivative neuroprotection D1 (NPD1) were shown by the investigators group at Louisiana State University (LSU). Ludmila Belayev tested post-stroke intravenous injection of DHA (22:6n-3) in rats subjected to two hours of middle cerebral artery occlusion (MCAO).² DHA significantly improved behavior outcomes and attenuated brain edema (MRI T2-weighted image) and infarct size up to seven days after stroke. Such improvements were seen when DHA was injected at five hours after onset of stroke (i.e. three hours after reperfusion). Enhanced NPD1 synthesis in the brain penumbra area in DHA-treated animals was demonstrated by lipidomic analysis.

Nicholas Bazan (LSU) presented his unique approach with the combination of aspirin and DHA. Aspirin alone had been shown to afford beneficial effects against cerebrovascular diseases. He found that aspirin and DHA co-treatment induced the synthesis of aspirin-triggered NPD1 (AT-NPD1) in the brain.³ Injection of AT-NPD1 sodium salt or methyl-ester at three hours after stroke onset was effective in improving outcomes in rats subjected to two hours of MCAO.

Alberto Musto (LSU) reported protection by NPD1 in a mouse status epilepticus model induced by pilocarpine. Post-status epilepticus NPD1 treatment reduced recurrent seizure frequency and improved electrophysiological outcomes, suggesting that neuroprotection by NPD1 could be at the post synaptic level.⁴

Another potential candidate for neuroprotectant was proposed by Byron Ford (Morehouse School of Medicine). Ford studied neuregulin-1 which had originally been identified as a growth factor at the neuromuscular junction. Carotid arterial injection of neuregulin-1 showed impressive infarct reductions and neurological improvements in rats.⁵ Zhenfeng Xu presented their recent attempt of genomic and transcriptomic approaches using rat brain samples after stroke. Ford also discussed his recent experience with a non-human primate model in searching for potential biomarkers for predicting stroke outcomes.

Mami Noda (Kyushu University) discussed her unique neuroprotection approach using molecular hydrogen. She tested drinking hydrogen-containing water in rodent models of Parkinson's disease⁶ and ischemic model of optic nerve. Although the hydrogen level in the brain was not influenced, and although the underlying mechanism remained to be studied, hydrogen-containing water drinking was protective against those pathological conditions. Since inhalation of hydrogen gas has been shown to protect against ischemic stroke in rats, hydrogen-containing water drinking may also protect against stroke.

Findings from recent clinical studies were also presented. Shunya Takizawa (Tokai University) reported the outcomes of their phase I study of intravenous granulocyte colony-stimulating factor (G-CSF) in ischemic stroke patients.⁷ The clinical study was based on their previous findings in mice that hematopoietic cytokines reduced infarct volume with improvements in motor and cognitive functions. According to the phase I study, G-CSF (150 and 300 µg/body/day) was safe and well-tolerated in patients after ischemic stroke. A phase II study is underway, testing G-CSF treatment 24 hours after onset.

Koji Abe (Okayama University) presented the outcomes of edaravone (Radicut®) in a retrospective study of 114 consecutive stroke patients who received tissue plasminogen activator (tPA, 0.6 mg/kg) within three hours after onset.⁸ Edaravone is free radical scavenger that was approved for treating acute ischemic stroke in Japan in 2001. Edaravone-treated patients showed a higher recanalization rate after tPA compared with those who did not receive edaravone although there was a caveat that edaravone-treated patients had a higher prevalence of cardiogenic embolism and lower NIHSS scores on admission.

Cerebrovasculature and neurovascular protection against ischemic stroke

Study of the cerebrovasculature and its interaction between the neurovascular unit are traditional and indispensable areas confronting stroke research. Recent advances in the pathophysiology of the BBB and novel approaches to cerebrovascular regulation were discussed.

Tetsuro Ago (Kyushu University) discussed the neurotrophic roles of pericytes. He showed that the expression of platelet-derived growth factor receptor β (PDGFR β) is drastically elevated in pericytes of the peri-infarct areas in mice after MCAO.⁹ Heterozygous PDGFR β gene knockout suppressed the elevation of PDGFR β with increased infarct formation. In addition, PDGF β , the ligand for PDGFR β , elevates the expression of nerve-growth factor (NGF) and neurotrophin-3 (NT-3) in cultured pericytes. These findings suggest that the PDGF β -mediated NGF/NT-3 production from pericytes may afford neuroprotection against ischemic stroke.

David S. Miller (National Institute of Environmental Health Science) described the importance of nuclear factor E2-related factor 2 (Nrf2) in drug delivery through BBB. Nrf2 is a redox-sensitive, ligand-activated transcription factor that induces multiple antioxidant and glutathione generating enzymes in response to oxidative stress. He assessed the effects of Nrf2 activation at BBB on drug efflux transporters, including P-glycoprotein.¹⁰ Nrf2 ligand sulfuraphane elevated protein expression of P-glycoprotein as well as the efflux activity in isolated rodent brain capillaries, suggesting that this pathway contributes to restricting drug delivery across BBB when Nrf2 is activated.

Jeffrey M. Gidday (Washington University) highlighted vascular mechanisms in ischemic tolerance. He demonstrated that hypoxic preconditioning reduced post-stroke leukocyte adhesion and BBB dysfunction. Downregulation of inter-cellular adhesion molecules and enhanced tight junction integrity, including ZO-1 and claudin-5, have been documented as a common phenotype in vascular ischemic tolerance.¹¹ A better understanding of the vascular effects of ischemic tolerance is warranted.

David Busija (Tulane University) summarized his research on vascular mitochondria as a therapeutic target. Transient activation of ATP-dependent potassium channels that are presented on the inner mitochondrial membrane (mitoKATP channels) induces immediate and long-term protection of cerebral endothelium against subsequent stress.¹² Attenuations in both intracellular calcium elevation and reactive oxygen species production after the stress are likely to induce this protection. In addition, many of the molecular consequences

after the mitoKATP channel activation have potential to influence cerebrovascular tone. These phenomena are blunted in insulin resistant Zucker obese rats, suggesting negative impacts of abnormal glucose/lipid metabolism, common comorbidities in stroke patients. Prasad V.G. Katakam (Tulane University) showed that mitochondrial activation also promotes neuronal isotype-mediated nitric oxide (NO) generation, suggesting the existence of a novel link between neuronal metabolism and vasodilatation.

A therapeutic approach targeting peroxisome proliferator-activated receptor (PPAR) α in cerebral vessels was discussed by Shobu Namura and Donghui Li (Morehouse School of Medicine). The PPARs are nuclear receptors which act as transcription factor. Fibrates, clinically-used drugs for dyslipidemia, are known to activate PPAR α . Fibrates improve cerebral blood flow (CBF) in the penumbral area.¹³ Fibrate-induced elevation of superoxide dismutase (SOD) activity in brain microvessels may contribute to maintaining NO bioavailability, with improvement of ischemic CBF.

Immune responses and ischemic stroke

Immune responses following stroke continue to be an active area of investigation. Innate and adaptive immune responses in stroke were discussed. Innate immune responses have been more extensively investigated, since acute neurological insults were not traditionally considered in the context of prior antigen exposure. Newer aspects of innate immunity were also discussed, as they relate to acute and long term effects of stroke. Adaptive immunity may be relevant in the search for a vaccine against stroke, and may also help to understand why concurrent infections are detrimental to stroke outcome.

Kyra Becker (University of Washington) reviewed the literature on adaptive immune responses in experimental stroke, and presented a new model of adaptive immunity, whereby the systemic administration of lipopolysaccharide (LPS) at the time of stroke led to Th1 responses and worsened outcome.¹⁴ This model could be likened to the negative outcomes in stroke patients with complicating infections. Interestingly, adoptive transfer of splenocytes primed towards a Th1 response led to a worsened outcome, whereas adoptive transfer of splenocytes primed towards a Treg response led to a better outcome. These data suggest that interventions preventing Th1 or enhancing Treg responses may have translational value.

A study of novel immune molecules in experimental stroke was presented by Dr. Ooboshi (Fukuoka Dental College). Prior work has shown that lymphocytes lead to worsened outcome. However, the role of T lymphocyte subtypes has not been well studied. The gamma-deltaT cells can produce IL-17 following stimulation by macrophage derived IL-23. These cytokines appear to contribute negatively to stroke evolution, as mice deficient in these cytokines are protected.¹⁵ Further, peroxiredoxin (Prx), an endogenous antioxidant, induces IL-23 and leads to worsened stroke outcome through toll-like receptors-2 and -4 and MyD88 pathway. These findings show that Prx is a novel damage-associated molecular pattern. Inhibiting Prx appears to be protective.

While many described immune responses to date have been shown to be largely detrimental in the acute phase, Midori Yenari (University of California, San Francisco, UCSF) described a newly characterized innate immune receptor as a potentially beneficial player. This receptor, triggering receptor expressed on myeloid cells-2 (TREM-2), is thought to trigger phagocytosis in microglia and macrophages. Its ligand has been identified on brain cells, and recent work has shown that neurons exposed to apoptotic insults lead to the activation of TREM-2.¹⁶ TREM-2 deficiency decreased phagocytosis of injured neurons. Masahito Kawabori (UCSF) then showed that the proportion of microglia with TREM-2 expression

appeared enhanced under conditions of therapeutic hypothermia. Thus, TREM-2 may contribute beneficial effects, such as the clearance of cellular debris.

The link between pain and inflammation is well known, and Nozomi Akimoto (Kyushu University) presented new findings showing how the CCL-1 cytokine enhances nociception and microglial activation.

Recovery and repair and modeling of post stroke fatigue

Since the discovery of endogenous neural stem cells in the adult brains, and since recent reports that post-stroke systemic administration of mesenchymal stem cells improved outcomes in animals, neuronal repair has rapidly emerged as a potential method for treating stroke. As the Nobel Prize in Physiology or Medicine 2012 was timely (one week before the workshop) awarded to Sir John B. Gurdon and Shinya Yamanaka for the discovery that mature cells can be reprogrammed to pluripotency, presentations concerning this topic were actively discussed.

Koji Abe (Okayama University) discussed his exploration of the potential of the induced pluripotent stem (iPS) cell transplantation as a novel therapy for ischemic stroke. Unexpectedly, intracranially transplanted iPS cells formed teratomatous tumors in the ischemic mouse brains and the clinical recovery from stroke was delayed, despite increases in the number of neuroblasts and mature neurons in the ischemic brains.¹⁷ He concluded that iPS cell therapy had a promising potential to provide neural cells after stroke if tumorigenesis could be controlled.

Optimal delivery method and timing of neural stem cell therapy against stroke were discussed by Raphael Guzman (Stanford University). He recommended that intravascular injection was advantageous over intra-parenchymal transplantation for achieving a widespread distribution while being minimally-invasive and repeatable. Compared to intravenous injection which often results in entrapment of transplanted cells in the lung, intra-arterial injection provides better homing into the brain. In terms of timing of the intra-arterial approach, injection at three days after ischemia resulted in the highest cell engraftment.¹⁸ Pretreatment of transplanted cells with BDNF enhanced the therapeutic efficacies.

Another recovery approach targeting angiogenic factor Netrin-1 was discussed by Jialing Liu and Chih Cheng Le (UCSF). Netrin-1 gene delivery into the ischemic penumbra not only increased vascular density but also promoted the migration of immature neurons into the peri-infarct white matter, which was accompanied by improved recovery of motor function.¹⁹ The enhanced neurogenesis by Netrin-1 was likely to contribute to the neurological recovery because conditional ablation of neuroprogenitor cells by targeting nestin in adult mice delayed the recovery of cognitive function after stroke without affecting CBF and subsequent lesion size.

Finally, Allison Kunze (University of Washington) presented a novel approach for detecting post-stroke fatigue in popular rodent stroke models. This approach should open up the field to the identification of new treatments for a significant but understudied clinical problem.

Novel imaging technologies for stroke studies

In vivo optic brain imaging is an emerging area in experimental stroke studies. For instance, as fluorescence labeling technique advances, two-photon excited microscopy provides tremendous potential of documenting repeatedly not only neurovascular morphology and

hemodynamics but also changes at the molecular level, including those in intracellular ion levels.

Chris Schaffer (Cornell University) discussed his experience in developing micro-scale stroke models utilizing femtosecond laser pulses.²⁰ By controlling the amount of the delivered energy, either vessel occlusion or rupture can be produced at a pin-point. Concerning neocortex micro-infarct, the location of the occlusion determines the consequence: in contrast to the sustained perfusion deficit in the distal portions after occlusion of a penetrating arteriole, there was a robust reversal flow from distal branches after occlusion of cortical surface arterioles. On the other hand, rupture of a penetrating arteriole (micro-hemorrhage) triggered local inflammatory responses without detectable neurovascular pathology, such as dendrite deformation and capillary collapse, in the surrounding tissue. Nozomi Nishimura (Cornell University) showed in a mouse model of Alzheimer's disease that capillary flow was dramatically stalled, which was accompanied by leukocytes adhesion to the endothelium. The capillary plugging by leukocyte may cause the CBF impairment in Alzheimer's disease.

Jialing Liu and Yosuke Akamatsu (UCSF) shared their experience with optical coherence tomography and optical microangiography for measuring cerebrovascular microstructure and flow, and post-ischemic collateral circulation in db/db mice, a mouse model of type 2 diabetes mellitus. Type 2 diabetes mellitus is known to be associated with worse stroke outcomes. Compared with non-diabetic db/+ mice, db/db showed lower regional CBF and lower density of functional blood vessels in the ischemic hemisphere. These new imaging methods are useful for monitoring collateral flow development in mice after stroke.

Overall summary and future directions

The meeting was viewed by most of the attendees as a successful start to developing collaborative efforts between the two countries to investigate the patho-mechanisms of ischemic stroke. The size and format of the meeting were well received, especially by the Japanese participants and junior investigators who often feel intimidated in speaking at large-scale conferences. The location and timing surrounding the Society for Neuroscience Meeting was convenient for the US participants, particularly basic researchers and students.

The meeting clearly demonstrated the need for future meetings that could expand on topics covered. For example, edaravone has been used in stroke patients in Japan, and US participants expressed an interest in learning more about Japanese clinicians' experience, in order to consider larger-scale international studies. Neural repair using iPS was another actively discussed topic. Due to their accessibility, pluripotency and autologous nature, iPS technology has a tremendous potential to treat neurodegenerative diseases, including stroke. Future investigations in applying iPS technology to treat stroke patients may be one area of collaboration between investigators in the US and Japan. Establishing standardized preclinical models and unbiased study methods would allow comparison of biological robustness of findings across laboratories, which is important when considering clinical translation. Related to this issue, developing a reproducible non-human primate model of stroke will certainly be useful to test promising neuroprotectants, such as neuregulin-1, DHA, and NPD1 that were discussed above. Collaborations with physicists and chemists would be needed as shown by the examples of in vivo imaging.

Acknowledgments

This meeting was funded by a NIH R13 grant through the US-Japan Brain Research Cooperative Program (3R01 NS040516-11S1) to Dr. Midori Yenari, The Japan-US Science and Technology Cooperation Program Brain Research Division to Dr. Hiroaki Ooboshi, Genentech (a member of the Roche Group), and Peter R. MacLeish

(Neuroscience Institute, Morehouse School of Medicine). Grant funds were administered by the Northern California Institute for Research and Education. The organizers of the “Trans-Pacific Workshop on Stroke” thank the presenters and attendees for their contributions and lively and insightful discussions. The attendees dedicated the meeting to the memory of Akira Arimura, an American scientist at Tulane University with beginnings in Japan, whose remarkable career included many US-Japan collaborations.

Koji Abe gave the featured lecture “Current topics on neuroprotection after cerebral ischemia.” The lecture provided a brief report on the impact of the unprecedented disaster caused by the earthquake/tsunami in March, 2011 on the life and health conditions of the affected individuals and on the health care service in the affected Tohoku Pacific Coastal areas in Japan. His report reminded the attendees that the workshop was taking place in a town that had been hit by Hurricane Katrina seven years ago. It was delightful to have the local speakers Nicolas Bazan, Ludmila Belayev, and David Busija who remain active in their forefront stroke research.

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Figure 1.

The ischemic cascade of events in the brain after ischemic stroke. Events are color coded according to their timing: red, acute phase (minutes to hours); green, subacute phase (hours to days); and blue, chronic phase (weeks to months). Cellular and molecular mechanisms that are highlighted in yellow were specifically featured by the workshop and discussed by the speakers.

Figure 2. Schematic representation of cellular events in the neurovascular unit and potential therapeutic targets that were discussed at the workshop. Endothelial cells (blue) of the cerebral vessels and basal lamina (purple) are surrounded by an almost continuous layer of astrocyte (red) foot processes. Pericytes (orange) also cover the abluminal surface of the capillaries. In addition to these structures, tight junctions and transporters that are expressed on the endothelial cells contribute to the blood–brain barrier (BBB), a unique feature of cerebral vessels. After ischemia, in addition to neuronal injury, damage and activation of endothelial cells leads to BBB disruption and extravasation of blood-derived cells and serum molecules. Within the brain itself, microglia, the brain’s resident immune cells, are activated. Endogenous molecules and responses, and protective agents are shown in blue text. Targets where inhibition is protective are shown in red text. (*, edaravone is already in use for treatment of ischemic stroke patients in Japan).