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Can Genes Modify Stroke Outcome and By What Mechanisms

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The approach to neuroprotection in stroke has relied mainly on exogenously administered drugs derived from research probing the cellular mechanisms of ischemic brain injury. This approach has had limited translational impact. Over 1000 putative neuroprotective agents have been developed, and over 100 have advanced to clinical trials. None, however, have shown clinical efficacy[1].

By contrast, a gene-based approach seeks to harness endogenous neuroprotective programs for stroke therapy. Experimentally, ischemic brain injury can be attenuated and stroke outcome improved by gene expression modification in brain. The brain and other organs have highly conserved, endogenous neuroprotective programs, the induction of which reduces ischemic injury. The neuroprotective programs can be induced in the model of ischemic tolerance: brief exposure to sublethal ischemia produces tolerance to a subsequent, severe ischemic challenge. A number of laboratories have taken this approach with differing mechanistic foci which have been reviewed elsewhere[2–5]. The protection induced by tolerance is substantial, gene-based, and dependent on new protein synthesis. The neuroprotection of tolerance has been demonstrated in experimental cardiopulmonary bypass surgery[6] and in stroke in the primate[7]. A clinical counterpart likely exists in human brain: patients with prodromal transient ischemic attacks have milder strokes[8–11]. Two small clinical trials have shown potential benefits of preconditioning, and five additional trials are underway[12]. Thus, there is considerable therapeutic potential in understanding gene expression changes in tolerance and in dissecting the biological mechanisms that regulate them. This is an evolving story deriving mainly from the authors' and our collaborators laboratories.

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Individual Genes

Over the last several decades, research has identified numerous genes whose regulation may affect the outcome of stroke. Because cell death following harmful ischemia is mediated by programmed cell death–like mechanisms, many studies of neuroprotection have focused on apoptotic mediators as potential effectors of tolerance to ischemia. The regulation of genes in cell death pathways, particularly those that affect mitochondrial integrity, has been studied extensively. Regulation of genes for caspases, Bcl family members, protein kinases, hypoxia inducible factor (HIF), and apoptosis-inducing factor (AIF)[13–16] can alter stroke outcome. For example, the use of antisense to block upregulation of the cell survival protein Bcl-2 during stroke results in a larger infarct, whereas inhibition of the cell death protein Bax reduces stroke volume. Numerous studies have also focused on heat shock proteins, which constitute a highly conserved, gene-based, response to stress. They act as chaperones and have anti-apoptotic and anti-inflammatory properties[17, 18]. Heat shock proteins are neuroprotective following both exogenous (viral vector gene transfer) and endogenous (transgenic) upregulation. Other highly conserved, widely distributed, gene-based systems with neuroprotective modulatory properties include inflammatory mediators and the toll-like receptor (TLR) system[19]. Finally, stem cell proliferation also appears to be neuroprotective in stroke[20]. Paracrine action of factors secreted from these cells is a suggested mechanism of action. Microarray analysis of gene expression in stem cells has identified subpopulations of bone marrow progenitor cells optimal for neuroprotection in stroke[21].

Genomics

Efforts to identify the molecular effectors of ischemic injury and tolerance relied for many years on a one-gene-at-a-time approach. In the past decade, genomic studies have provided a more complete picture of gene regulation in stroke. The first application of unbiased mRNA screening to the study of endogenous neuroprotection was performed in a mouse model of ischemic tolerance[22]. The microarray analysis of ischemic tolerance proved a powerful tool for gene discovery and demonstrated that the genomic profile of protection can be determined. The analysis showed that scores to hundreds of genes are regulated after ischemic preconditioning, injury, and tolerance. Additionally, the broad view of gene regulation provided by the microarray analysis offered new insight into mechanisms of stroke neuroprotection. The genes regulated after preconditioning ischemia and the genes regulated after injurious ischemia are remarkably different. Moreover, genes regulated in ischemic tolerance are distinct from those regulated after preconditioning or injurious ischemia. Most notably, regulated genes were predominantly induced in injury but suppressed in tolerance (Figure 1)[22].

The results from microarray studies of ischemic tolerance underpinned a novel hypothesis about the neuroprotective mechanism: it was proposed that preconditioning reprograms the brain's response to ischemic challenge, altering the transcriptional response from one that leads to cell death to one that produces a neuroprotected phenotype[22]. Gene suppresion was hypothesized to be a central neuroprotective feature of tolerance. Among suppressed genes, those that encode ion channels, transporters, and metabolic pathways are particularly affected, reminiscent of changes that allow hibernating animals to survive periods of prolonged oxygen and glucose deprivation[23, 24].

The hypothesis that the brain's response to injury can be reprogrammed is supported by genomic profiling in epileptic tolerance. As in ischemic tolerance, a brief noninjurious seizure preconditions the brain so that it is tolerant to subsequent challenge by a prolonged, injurious seizure. Transcriptional changes that occur in the susceptible hippocampal CA3 subfield in mouse have been profiled after seizure preconditioning, epileptic challenge, and

epileptic tolerance [25, 26]. Similar to ischemic tolerance, preconditioning seizures[25, 27, 28] produce a different pattern of gene expression than do injurious seizures[29–31]. Further, the response to injury is reprogrammed: the set of genes regulated after prolonged seizures differed from the set of genes regulated after prolonged seizure that was precedeced by a preconditioning seizure to produce epileptic tolerance. Consistant with ischemic tolerance, the majority of genes differentially regulated in the tolerant brain were suppressed[26]. In contrast to ischemic tolerance, genes suppressed in epileptic tolerance encode proteins that participate in calcium signaling and excitatory neurotransmission[26]. Therefore, the genomic signature of neuroprotection seems to be specific to the stress, as has been previously suggested[32]. Seizure-preconditioning specifically promotes an anti-excitotoxic phenotype—a phenotype apposite to the inducing stimulus—just as lipopolysaccharide (LPS) preconditioning produces an anti-inflammatory phenotype and preconditioning ischemia produces a hypo-metabolic phenotype[32]. Of note, these phenotypes are appropriate to the nature of the preconditioning stimulus and not necessarily to the nature of the challenging stimulus.

As an endogenous neuroprotective mechanism, tolerance can be understood as a first insult priming the brain to respond advantageously in the likelihood of a second insult of the same kind. Yet the preconditioned brain appears also to respond advantageously to a second insult of a different kind[33, 34]. The basis for this is not yet clear. Microarray analyses have made it apparent that the response to any brain challenge is complex, engaging numerous and diverse pathways. There may also be shared neuroprotective mechanisms not detectable at the transcriptional (mRNA) level. Uncovering those mechanisms will advance our understanding of endogenous neuroprotection and facilitate therapeutic intervention.

Transcription factors

Transcription factors transduce intracellular signaling cascades into genomic and proteomic responses following preconditioning. To identify transcription factors that may coordinate the reprogrammed neuroprotective response, genomic studies compared gene regulation in ischemic tolerance induced by three different preconditioning agents: lipopolysaccharide (LPS, a TLR4 ligand), unmethylated CpGs (a TLR9 ligand), and brief ischemia. The studies identified 13 genes regulated in all three models of ischemic tolerance (Figure 2, left). Bioinformatic analysis of these genes' promoter regions identified transciption response elements for interferon regulatory factors (IRF) (Figure 2, right), suggesting that a set of genes is commonly and coordinately regulated in the neurorprotective response to ischemia. Consistently, preconditioning-induced tolerance to ischemia was abrogated in mice deficient in IRF3 and IRF7[35]. Promoter analysis of genes regulated in all three different models of tolerance supports the view that different preconditioning stimuli activate common neuroprotective mechanisms.

microRNAs

While extrapolation from microarray profiles of tolerant brain has identified a few upstream transcription factors and signaling pathways[34] that may coordinate the genomic response in tolerance, the mechanisms at work during the development of the neuroprotected state, between preconditioning and challenge, have not been defined. How does the preconditioning stimulus prepare the brain to respond to subsequent stress in a new, reprogrammed, and protetective manner? Recent work suggests that microRNAs (miRNAs) may play a pivotal role in the molecular response to the preconditioning stimulus[36]. MiRNAs are short (~22 nucleotide) endogenous, non-coding sequences of RNA that post-transcriptionally regulate gene expression in plants, animals and viruses[37–40]. Through complimentary base-pairing interactions, miRNAs recognize target mRNA transcripts and

direct their incorporation into the RNA-induced silencing complex (RISC), leading to a decrease in protein expression, either through translational repression or mRNA decay (for review [41–43]). Recently, miRNA expression was profiled in ischemic preconditioning, ischemic injury, and ischemic tolerance[36]. Marked regulation of miRNA expression was observed in preconditioned brains, whereas little was observed in ischemic or tolerant brain (Figure 3A)[36]. Among the most prominent targets of the miRNAs regulated in preconditioned brain was methyl CpG binding protein 2 (MeCP2), a global regulator of gene transcription. Consistent with decreased expression of miRNAs targeting MeCP2, MeCP2 protein was increased in preconditioned brain (Figure 3B). Loss of MeCP2 precluded the induction of tolerance (Figure 3C), thus confirming the importance of this miRNA-mediated pathway[36]. Accordingly, miRNA profiling of ischemic brain supports the proposition that the preconditioning stimulus regulates miRNAs that target transcription factors and thereby lead to differential gene expression which characterizes tolerant brain. In the case of MeCP2, the effect is to repress transcription.

Proteomics

Genomic studies of neuroprotection have shown that the responses to ischemia are complex and wide-ranging. Understanding of the molecular mechanisms involved therefore continues to evolve. At the genomic level, the induction of neuroprotection entails a repressive transcriptional response. Identifying the actuators of transcriptional suppression would move us closer to the goal of activating neuroprotection clinically. Recent proteomic studies of ischemic tolerance suggest that these actuators may be epigenetic regulatory proteins[44].

The proteome of ischemic tolerance in rodent was characterized using an unbiased, quantitative proteomic approach, followed by biochemical and physiological studies[44]. The results fit nicely into the concept that the phenotype of neuroprotection is one of transcriptional suppression. Epigenetic silencers, including polycomb group (PcG) proteins and modified histones, are enriched during ischemic tolerance in the brain. Experimentally, changes in PcG protein levels are sufficient to induce or inhibit the tolerant state in neurons. Thus, knockdown of PcG proteins blocks the neuroprotective response, whereas over-expression induces the neuroprotective response. Additional evidence indicates that other epigenetic proteins may also be involved[44]. A similar biology occurs in ischemic tolerance in retina[45].

Polycomb group (PcG) proteins and their antagonists, trithorax group (TrxG) proteins, although known to be present in brain[46], had not previously been implicated in neuroprotection. We hypothesize that these proteins, along with their partners, are "master regulators" that switch the mammalian central nervous system neurons from a stress-sensitive (unprotected) to a stress-resistant (protected) state. In *Drosophila*, where they were originally identified as developmental regulators[47], PcG and TrxG proteins alter gene expression by epigenetic means, maintaining over 1000 genes in an active or repressed state[48, 49]. Studies of PcG proteins in other systems show that they target a wide range of genes, including those regulated in ischemic tolerance, such as potassium channels, which are repressed during tolerance[22, 48–51]. Recent genome-wide screening of PcG targets in human embryonic fibroblasts identify cellular pathways that PcG proteins may regulate controlling development, differentiation, stem cell biology, and cell fate decisions [48]. Notably most of the pathways described are known to be involved in the brain's response to ischemia. Together, these data suggest that epigenetic regulation is a central mechanism for the induction of ischemic tolerance and that PcG proteins may be the key actuators. An endogenous neuroprotective mechanism mediated by PcG proteins explains many of the genomic and physiologic characteristics of ischemic tolerance in the brain. It is also perhaps a more general regulator of cell fate, as suggested by Suzanne Zukin[52].

Epigenetic mechanisms control gene expression by remodeling the architecture of chromatin in ways that allow or deny transcription factors access to genes. PcG proteins interact with DNA at polycomb response elements (PRE) to silence the expression of genes, including those that encode electron transporters, endopeptidases, oxidoreductases, and G-protein coupled receptors[51, 53, 54]. For many genes, silencing by PcG proteins is countered by activation by trithorax group (TrxG) proteins. Figure 4 illustrates a simplified model of epigenetic regulation by PcG and TrxG proteins.

PcG proteins assemble into at least three major complexes, each with a distinct role in epigenetic regulation, that work in concert with one another. The composition of these complexes is dynamic and influential in determining the outcome of biological processes, such as cell fate determination[55, 56]. Polycomb repressive complex 1 (PRC1), for example, mono-ubiquitinates histones H2A and H2B, whereas PRC2 methylates histone H3. In studies of ischemic tolerance, in rodent brain and neuronal cultures, three PcG proteins - Scmh1, Bmi1, and Ring1B – are robustly upregulated. SCMH1 acts as a link between PRC1 and other proteins[57]. Bmi1 facilitates monoubiquitination of histone 2A[58]. Ring1B functions as a ligase in H2a ubiquitination [58]. Other proteins that comprise epigenetic regulatory complexes may also be differentially regulated during tolerance. The TrxG protein, ASH1L, is in fact downregulated in ischemic tolerance consistent with the counteracting roles of TrxG and PcG proteins.

Thus, the emerging picture is that PcG and TrxG proteins undergo dynamic regulation during the induction of ischemic tolerance in the brain. This changes the composition of PcG and TrxG complexes, alters the ratio of silencing to activating complexes, and ultimately modulates the expression of target genes. PcG protein levels increase during tolerance, very early after ischemia, suggesting that this response initiates the neuroprotective cascade. The upregulation of PcG proteins during tolerance occurs, at least in part, via a transcription-independent, translational mechanism. Emerging evidence indicates that microRNAs regulate PcG protein expression[59, 60].

Conclusion

The discovery that the brain's response to injury can be governed by epigenetic modulation of gene expression offers new insight into mechanisms of brain injury and protection. Specifically, the discovery that PcG /TrxG proteins are active in brain ischemia reveals that an evolutionarily conserved mechanism is active during ischemic stress. This mechanism maintains chromatin in an on or off state, promotes or suppresses gene transcription, and thereby affects cell fate.

Studies of oncogenesis already implicate the PcG/TrxG system as a potent cell fate regulatory system in humans [61]. For example, the polycomb group protein EZH2, encoded by the *EZH2* gene on chromosome 7q36, functions as a gene repressor. Altered expression of EZH2 occurs in a number of malignances including prostate cancer, where EZH2 knockdown inhibits proliferation of prostate cancer cells [62]. In light of this, PcG proteins may be viewed as a prolife signal: turned on transiently, as in tolerance, it promotes cell survival; turned on continuously, as in cancer, it ultimately causes malignancy.

Epigenetic proteins, whose role in neuroprotection was previously unknown, may be “master regulators” of a neuroprotective state in the mammalian brain. Based on our findings that 1) the genomic signature of ischemic tolerance is transcriptional suppression and 2) the proteomic signature of ischemic tolerance is enrichment of epigenetic gene silencers, we submit that widespread changes in gene expression, coordinately modulated by epigenetic regulatory proteins, can modify stroke outcome. In this “omic” view, therapeutic

approaches that target a discrete pathway appear of limited value. Thus, in hindsight it is not surprising that clinical trials based on this approach have failed. By contrast, a therapeutic approach that targets the PcG/TrxG system could initiate a comprehensive neuroprotective response in the brain.

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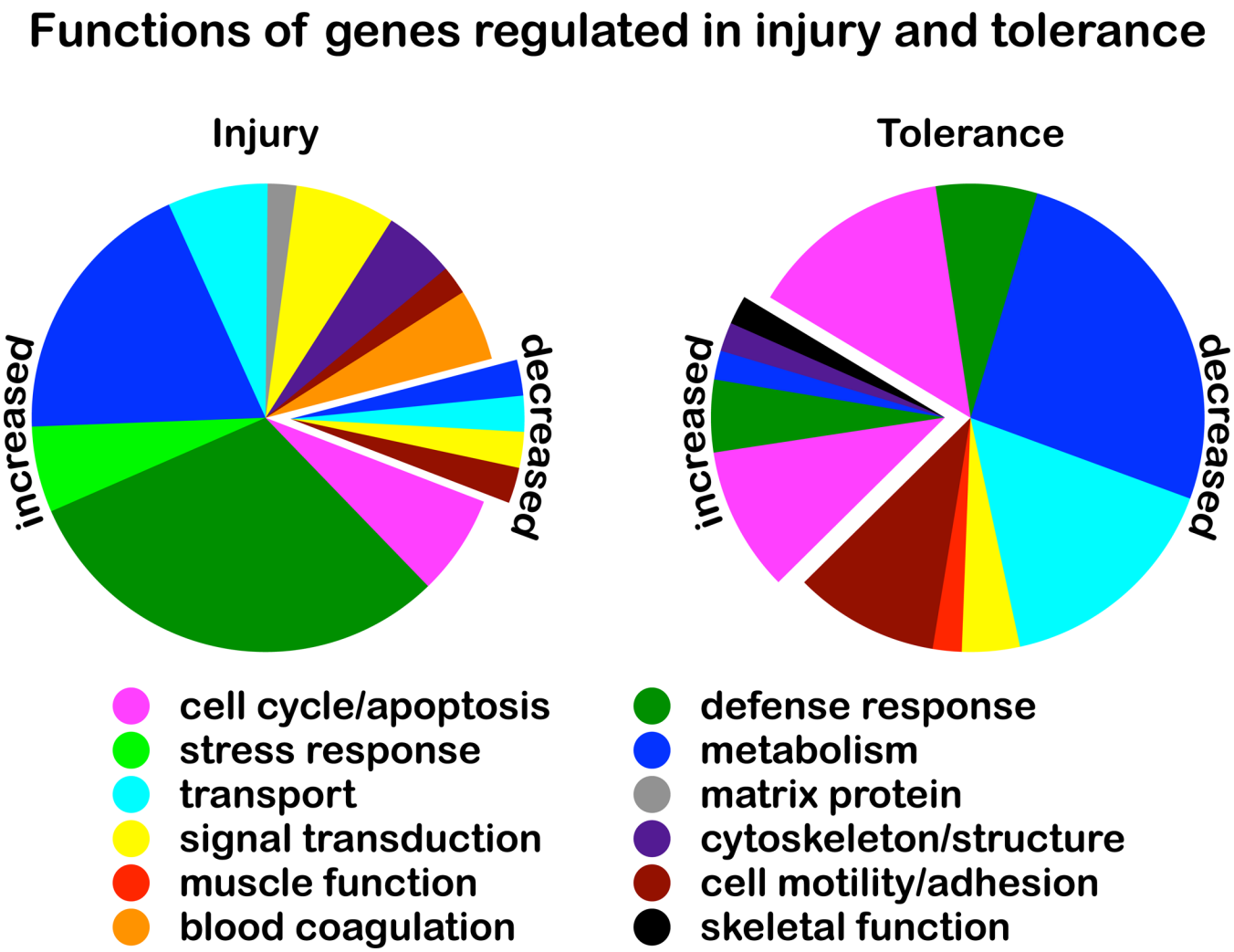

Figure 1.
Genes whose expression is uniquely increased/decreased in ischemic injury (left) or uniquely increased/decreased in ischemic tolerance (right) are categorized by biological function. In injury (stroke), a majority of regulated genes is expressed at higher levels. By contrast, in tolerance (protection), a majority of regulated genes is expressed at lower levels. Based on data from Stenzel-Poore et al, 2003.

Symbol	Title
Fcgr1	Fc receptor, IgG, high affinity I
HGF	Hepatocyte growth factor
Ifit1	IFN-induced protein with tetratricopeptide repeats 1
Ifit3	IFN-induced protein with tetratricopeptide repeats 3
Oasl2	2′-5′Oligoadenylate synthetase-like 2
Plscr2	Phospholipid scramblase 2
Pycard	PYD and CARD domain containing
Pyhin1	Pyrin and HIN domain family, 1
Viperin	Radical *S*-adenosyl methionine domain containing 2
Rtp4	Receptor transporter protein 4
Trim30	Tripartite motif protein 30
Usp18	Ubiquitin specific peptidase 18
Zbp1	Z-DNA binding protein 1

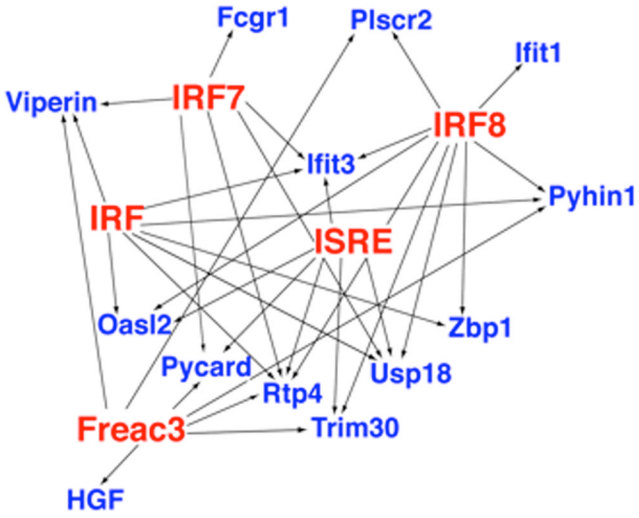

Figure 2.

Analysis of genes regulated in stroke preceded by preconditioning with ischemia, lipopolysaccharide (LPS), or CpGs. **Left**: Thirteen genes were regulated in all three conditions. **Right**: Promoter analysis identified five over-represented transcription response elements (TREs; red) that putatively regulate expression of the thirteen genes (blue). Four of the TREs (IRF, IRF7, IRF8, ISRE) associate with interferon signaling. Modified from Stevens et al, 2011 with permission.

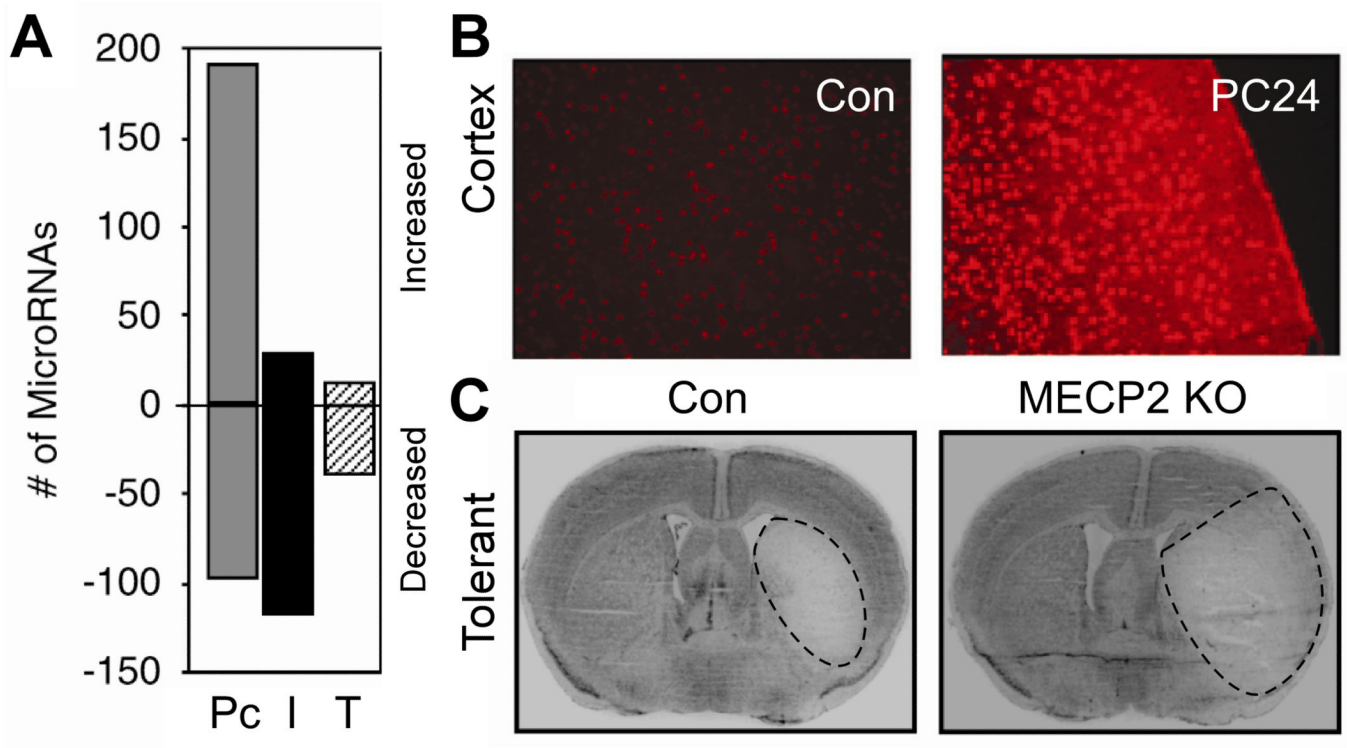

Figure 3.

MiRNAs in ischemic tolerance. **(A)** MiRNA screening reveals that the predominant response of miRNAs after preconditioning (P) is upregulation, versus downregulation in ischemia (I) and tolerance (T). **(B)** MeCP2 (methyl-CpG binding protein) immunostaining shows induction in the cortex 8 h after preconditioning, consistent with decreased expression of MeCP2 miRNA. **(C)** Infarct is larger in tolerized MeCP2-null mice than in tolerized wildtype (WT) mice. Adapted, with permission, from Lusardi et al, 2010.

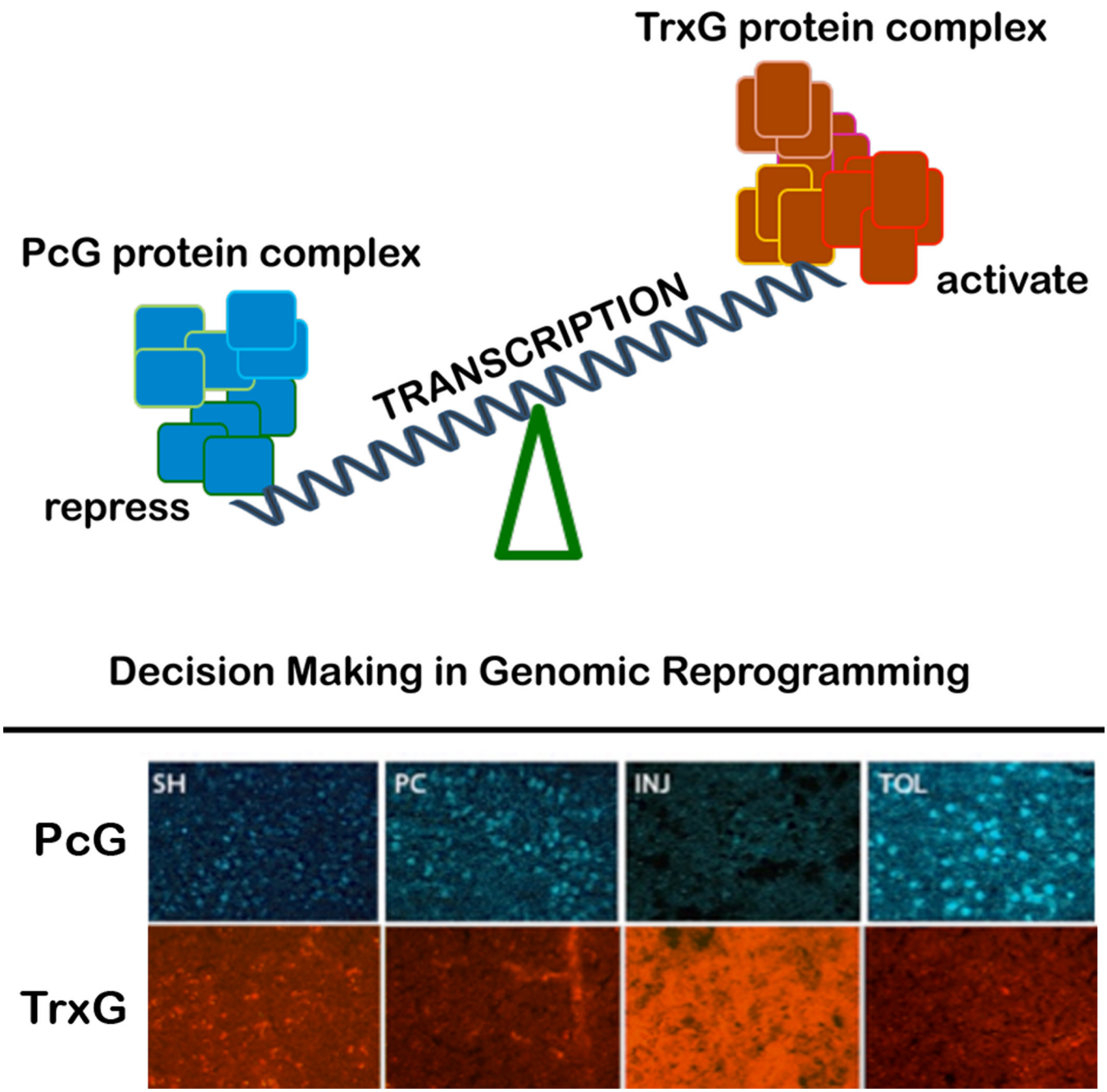

Figure 4.

Top: Proposed bivalent, epigenetic mechanism for regulating the brain's response to ischemia. Increased PcG protein abundance suppresses transcription. Increased TrxG protein abundance activates transcription. **Bottom**: In tolerance (TOL), PcG proteins increase. In injurious ischemia (INJ), TrxG proteins increase. The balance between PcG and TrxG activity may determine the response to ischemia. Transcriptional suppression and tolerance may be induced increasing PcG proteins, decreasing TrxG proteins, or a combination thereof.