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ORIGINAL ARTICLE

Protein Modifications with Ubiquitin as Response to Cerebral Ischemia-Reperfusion Injury

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Abstract Post-translational protein modifications present an elegant and energy efficient way to dynamically reprogram cellular protein properties and functions in response to homeostatic imbalance. One such protein modification is the tagging of proteins with the small modifier ubiquitin that can have an impact on protein stability, localization, interaction dynamics, and function. Ubiquitination is vital to any eukaryotic cell under physiological conditions, but even more important under stress including oxidative, genotoxic, and heat stress, where ubiquitination levels are drastically increased. Elevated levels of ubiquitin-protein conjugates are also observed in the brain after focal and global cerebral ischemia. Post-ischemic ubiquitination is immediately induced with reperfusion and transiently detected in neurons with survival potential located in the peri-infarct area. This review aims to critically discuss current knowledge and controversies on protein ubiquitination after cerebral ischemia, with special emphasis on potential mechanisms leading to elevated ubiquitination and on target identification. Further, possible functional implications of post-ischemic ubiquitination, including a relationship to SUMOylation, a neuroprotective modification, will be highlighted. The elevation in ubiquitinated proteins following cerebral ischemia is a greatly under-explored research area, the better understanding of which may contribute to the development of novel stroke therapies.

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Introduction to Ubiquitin

Ubiquitination, a Multifaceted and Complex Post-translational Modification

Ubiquitination is a highly versatile post-translational protein modification in all eukaryotic cells that occurs when the small 76 amino acid polypeptide ubiquitin is covalently attached to a target protein. It is estimated that there are more than 5000 proteins ubiquitinated in a cell at any given time, which makes up about one quarter of the encoded human proteome [1]. Therefore, the implications of ubiquitination on cell homeostasis are predicted to be enormous. Upon its discovery in the early 1980s, it was believed that ubiquitination solely serves as protein degradation signal [2, 3]. However, over the years, it became clear that it is essential for a wide variety of cellular processes, including cell cycle control, enzyme activation, signal transduction, transcription and DNA repair, receptor trafficking, immune response, inflammation, and apoptosis (reviewed in [4–8]). The remarkable ability of ubiquitin to influence protein function in seemingly endless ways stems on the one hand from the complexity of the conjugation system, and on the other hand from ubiquitin's ability to form polymers with different topologies (Fig. 1).

For the conjugation of ubiquitin to substrates with few exceptions, the consecutive action of three enzymes is required. In the first step, an E1-activating enzyme carries out the ATP-dependent activation of ubiquitin by forming a thiolester bond with its C-terminal glycine. Ubiquitin is then sequentially transferred to E2-conjugating and E3-ligating

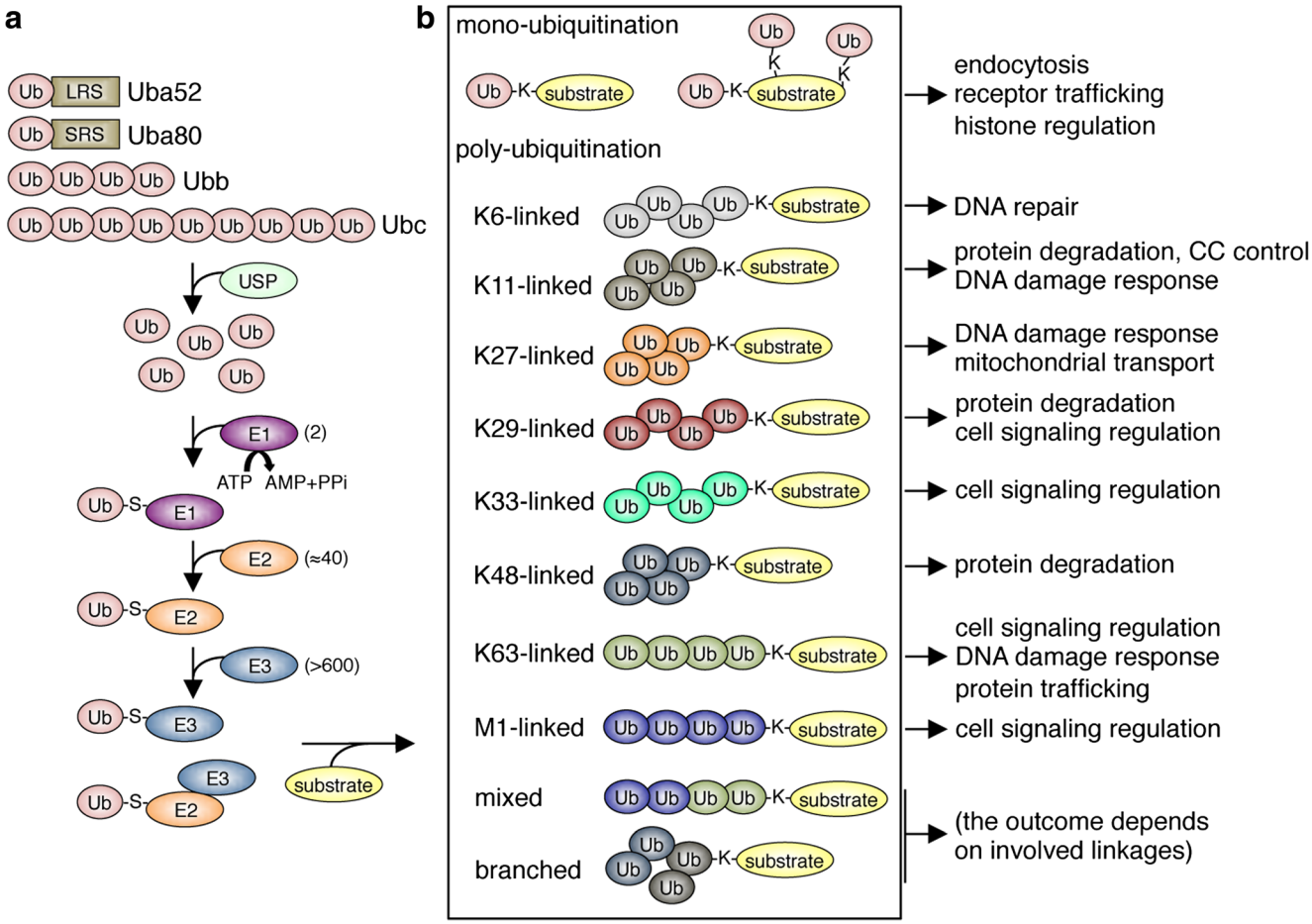

Fig. 1 The ubiquitin conjugation system is complex, influencing proteins in many different ways. **a** Mammalian cells, including neurons, contain four ubiquitin genes (Uba52, Uba80, Ubb, and Ubc) that contribute to the cellular ubiquitin protein pool. Ubiquitin is synthesized either fused to large (LRS) and small (SRS) ribosomal subunits, or as chains. Single ubiquitin molecules are obtained from these precursors by cleavage with ubiquitin-specific proteases (USPs). In order to be attached to a substrate, ubiquitin needs to be first activated in an ATP-dependent manner by an E1 activating enzyme, before it is transferred to an E2 conjugating and E3 ligating enzyme, with all of which it forms thiolester bondages (S). Finally, ubiquitin is covalently linked to a substrate by an isopeptide bond involving a lysine residue (K) in a substrate and a C-terminal glycine on ubiquitin. Whereas there are only two E1 enzymes and a restricted number of E2 enzymes (about 40), ubiquitin ligases are abundant (more than 600). An intimate interplay between E2 and E3 enzymes guarantees specific substrate recognition. **b** Ubiquitin can be attached to a target either as monomer, multiple monomers, or as chain that are formed through internal lysine (K) or the N-terminal methionine (M) residues in the ubiquitin molecule itself. Depending on the type of ubiquitin attachment, the functional outcome involving the substrate is entirely different (see list on the left). *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *CC* cell cycle, *PPi* pyrophosphate, *Ub* ubiquitin

enzymes, which catalyze the attachment of ubiquitin via an isopeptide bond to one or more lysine (K) residues in a protein substrate (reviewed in [9]) (Fig. 1a). While there are only two E1 enzymes encoded in mammalian cells, there are about 40 E2s and more than 600 E3s that have the potential to work together in a "mix and match" fashion to create about 25,000 different conjugation pairs that drive substrate specificity. In comparison, phosphorylation is directed by only about 520 kinases [10, 11]. Further, more than 100 deubiquitinating enzymes (DUBs) that can remove ubiquitin from a substrate have also been identified [12] (Fig. 2).

Apart from the increased number of conjugation options, ubiquitination has another additional layer of complexity compared with phosphorylation that greatly increases its versatility: it can form chains that are connected by each of its seven internal lysine residues (K6-, 11-, 27-, 29-, 33-, 48-, and 63-chains) and its N-terminal α-amino group (linear chains), resulting in at least eight different linkages [13, 14] (Fig. 1b). Although less commonly, mixed linkages as well as branched mixed chains, where different lysines in a single ubiquitin molecule are used for chain extension, are also observed [15–18]. Finally, ubiquitin can also be attached as a monomer on one or multiple target sites within a substrate (reviewed in [19]) (Fig. 1b). The fate of an ubiquitinated protein is critically dependent on the configuration of the associated ubiquitin (Fig. 1b). Well-characterized modifications in this regard are monomers as well as linear and K11, 48, and 63-linked chains. K48 chains with a few exceptions always

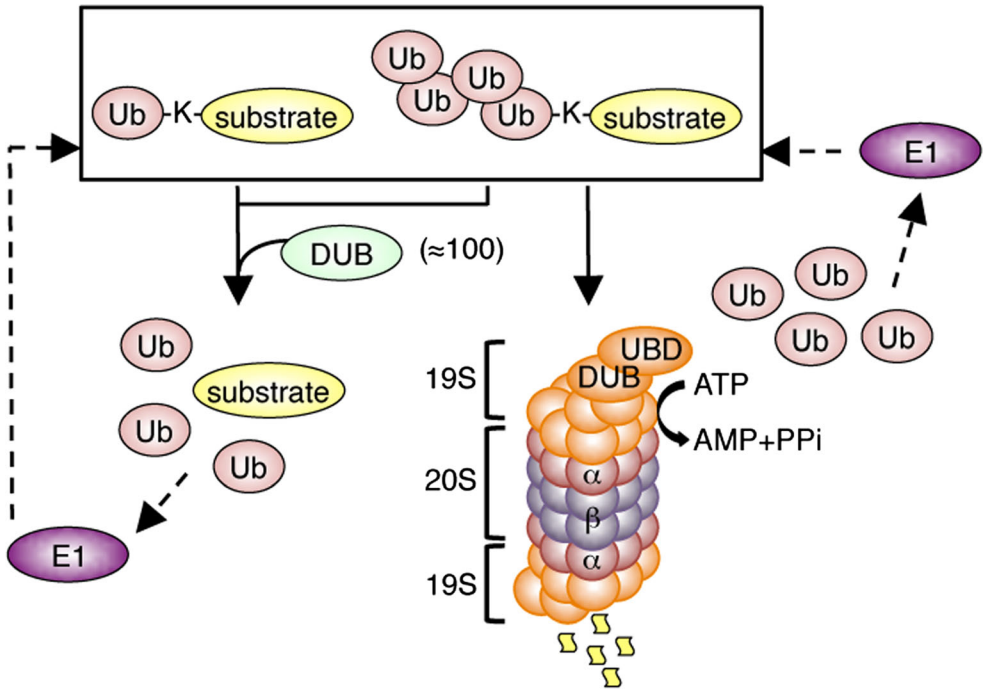

Fig. 2 The 26S proteasome, ubiquitin de-conjugation, and ubiquitin recycling. After serving their purpose, ubiquitinated proteins can be de-ubiquitinated by one of the approximately 100 known deubiquitinases (DUBs). On the other hand, if the attached ubiquitin chain signals for degradation, substrates are recognized and degraded by the 26S proteasome. The 26S proteasome consists of a 20S barrel-shaped core (α and β subunits) that has chymotryptic, tryptic, and caspase-like protease activities. The core is flanked on one or both sides with 19S lids that **a** recognize ubiquitinated proteins through ubiquitin-binding domains (UBDs), **b** remove ubiquitin from substrates via DUB activity, and **c** unfold substrates with AAA ATPase activity. De-ubiquitination by proteasome-dependent and -independent DUBs feeds ubiquitin molecules back into the free ubiquitin pool, awaiting a new conjugation round. *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *K* lysine residue, *PPi* pyrophosphate, *Ub* ubiquitin

serve as degradation signal, and there is some evidence that K11 and 29-linked chains serve as proteasomal recognition signals as well [20, 21]. On the contrary, monomers, K63-linked, and linear chains are completely non-degradative (reviewed in [22]). This is made possible by the entirely different three-dimensional shape of K11, 29, and 48 polymers compared to linear and 63-type chains [23–26], and naturally monomers. With this in mind, monomeric ubiquitination as well as ubiquitination via different chains may be seen as entirely distinct modifications that dictate a proteins fate in very different ways (Fig. 1b). This diversity of effects on proteins should also be considered when assessing ubiquitin's impact on the post-ischemic brain.

Ubiquitin-Mediated Degradation and the 26S Proteasome

Quantitative analysis revealed that K11, 29, and 48 ubiquitin chains represent about 60% of all ubiquitin linkages in vivo [21]. Although this analysis was carried out in yeast and the percentage may slightly vary in other eukaryotic cells, it can be predicted that about 50% or more of ubiquitinated proteins are tagged for degradation. The main protease responsible for intracellular protein degradation is a large 2.5 MD complex termed the 26S proteasome (Fig. 2). It consists of a 20S proteolytic core that is capped on one or both ends by a 19S/PA700 regulatory activator complex. The 20S core is built as a barrel-shaped ring structure containing two different types of subunits that have distinct structural and functional roles [27, 28]. The outer two rings are composed of α subunits that ensure structural integrity of the core, while the β subunits are forming the inner rings that confer proteolytic activity (Fig. 2). The 20S core by itself is essentially inactive as its structure sequesters the catalytic sites in an internal chamber that can only be reached through a narrow opening in the α subunits [27]. Although a role for the 20S core in the degradation of damaged or unfolded proteins has often been suggested especially in the context of oxidative stress [29, 30], it remains unclear as to how polypeptides manage to enter the uncapped 20S particles in vivo. Access to the 20S core is gated by the 19S/PA700 regulatory activator that confers specificity of the protease for ubiquitinated proteins by binding to ubiquitin chains and tethering the ubiquitinated substrate to the proteasome [31, 32]. The 19S particle contains a lid composed of subunits with ubiquitin-binding domain (UBD) and deubiquitinase (DUB) activities, and a base that contacts with the core particle and is composed of six ATPases, which belong to the AAA ATPase family (Fig. 2). Before delivering proteins for degradation to the 20S core, 19S/PA700 subunits

deubiquitinate [33, 34] and then unfold substrates in a highly ATP-dependent manner [35]. It should be mentioned that the 20S core can also associate with two alternative activators, the 11S/PA28 and PA200 complexes; however, these assemblies are unlikely to play a role in ubiquitin-mediated degradation as neither PA28 nor PA200 recognize ubiquitinated proteins or use ATP [36, 37].

Rather than being an unregulated destructive machine that uniformly degrades ubiquitinated substrate proteins, the activity of the 26S proteasome can be modulated by metabolic and inflammatory factors. Exposure to immune mediators or oxidative stress can initiate the exchange of classical proteolytic subunits of the 20S core with unconventional subunits that alter proteolytic capacity [38, 39]. This gives rise to the so-called immunoproteasome. This specialized form of the proteasome is mainly present in immune cells; however, it is also detected in non-immune cells, including the brain. However, while its role in immune cells with the generation of antigens for MHC class I presentation is well described (reviewed in [40]), it is not clear how it affects for example neurons or glia. Notably, there is indication that induction of the immunoproteasome in brain cells is associated with neurodegeneration [41–43]; however, it is not known whether this is a cause or effect of degeneration. Another means of altering 26S proteasomal activity is the post-translational modification of both 20S and 19S/PA700 subunits. In the brain, phosphorylation of Rpt6, an AAA ATPase member of the 19S complex, by CaMKII boosts proteasome activity to establish synaptic plasticity and memory [44–46]. Phosphorylation of the same subunit by PKA increases brain proteasome activity, and its associated down-regulation was proposed to be critical for Huntington's disease pathogenesis [47, 48]. An increase of O-GlcNAcylation and reactive oxygen species (ROS) in the brain is connected with reduction in proteasomal activity through modification of Rpt2 and Rpt3 proteasomal subunits [49–51]. Further, proteasomal activity is activated by poly-ADP ribosylation and acetylation of unidentified core subunits [52, 53], while ubiquitination of Rpn10 and Rpn13 19S subunits reduces proteolysis [54, 55]. Whether these modifications play a role in brain cells under stress, particularly after ischemia is yet to be determined.

Non-degradative Functions of Ubiquitin

Approximately 50% of ubiquitinated proteins are estimated to be associated with non-degradative ubiquitin chains or ubiquitin monomers [1] that control a variety of cellular functions primarily by modifying protein interaction profiles and protein activity. First, attached ubiquitin can promote the recruitment of new binding partners that are not at all or only weakly bound by the non-modified protein. An example for this in neurons is the ubiquitination of PSD95 by Mdm2, which does not affect PSD95 protein levels, but rather increases its interaction with β-adaptin to regulate AMPA receptor endocytosis [56]. Conversely, ubiquitin can also impede interactions. Monoubiquitination of the transcription factor Smad4, whose activity is important for neuronal development, blocks its association with the transcriptional co-activator Smad2 leading to blunted transcription [57]. Ubiquitination of receptors guides their endocytosis, sorting, recycling, and degradation by serving as docking signal for adaptor proteins. Prominent examples for this regulation in neurons are the AMPA and GABA receptors that require ubiquitination to initiate internalization and lysosomal degradation [58, 59]. Ubiquitination has also gained special attention as platform for the formation of signaling complexes and regulation of kinase function, which has been extensively shown in pro-inflammatory NF-κB signal activation [60–63]. In summary, in addition to playing a major role in protein degradation, ubiquitin serves also many important non-degradative functions that need to be kept in mind when considering the role of ubiquitination in brain cells under stress, e.g., after ischemia.

Ubiquitin Is Essential for Neuronal Development and Function

Ubiquitination is crucial for health and function of neurons. Synaptic plasticity and transmission, maintenance of synaptic strength, as well as memory formation are only a few examples that rely on a functional ubiquitination system [64–66]. It has been shown that increases and decreases in neuronal activity correlate with the amount of ubiquitinated proteins in dendritic spines [64]. Many key molecules involved in synaptic function are regulated by ubiquitination. These include structural components of the postsynaptic density, for example PSD95 [56, 67], GKAP [68], and Shank [64], or receptors like the AMPAR [58, 69], NMDAR [70], and GABAR [59, 71]. The effect of ubiquitination on the synapse is multifaceted. On the one hand, ubiquitination of scaffolding proteins strengthens the synapse, maintains synaptic morphology, and leads to reorganization of signaling complexes [67, 68]. On the other hand, ubiquitination of receptors leads to changes in receptor surface abundance, ensuring proper propagation of stimulatory and inhibitory signals [58, 70, 71]. In addition to its role in mature neurons, the ubiquitin system is also important during neurodevelopmental processes, such as dendrite pruning and axon guidance [72, 73].

Ubiquitin and the Stress Response

Apart from controlling important physiological functions, the ubiquitin system takes on essential roles in response to cellular stress. Indeed, an increase in the level of ubiquitin conjugates occurs with virtually all types of stressors, e.g., heat shock, oxidants, heavy metals, DNA damaging agents, etc. This is in

part made possible by an increased expression of ubiquitin itself. Mammalian cells, including neurons, contain four genes that feed into the cellular ubiquitin protein pool. The transcription of two of them, Ubb and Ubc, is heavily induced by stresses [74, 75], which provides an increased amount of ubiquitin necessary for survival under these conditions. Also, the activity and expression of E1 and some E2 and E3 enzymes are elevated as stress response [76–79]. The protection of cells via ubiquitin is mediated by degradative and non-degradative mechanisms. A stressed cell contains an increased amount of unfolded proteins due to changed energy demands that, if chaperones are overwhelmed with refolding, are tagged with predominantly K48-linked ubiquitin and delivered for degradation by the 26S proteasome. The role of K48-linked ubiquitination and proteasomal degradation in protein quality control is the topic of many expert reviews and will therefore here not be further discussed (reviewed in [80–83]). On the other hand, a vastly under-studied area is the role of non-degradative ubiquitin chains in stress response. Although not fully appreciated, it was already shown in 1994 that overexpression of ubiquitin unable to form K63 chains as opposed to all other chains sensitizes yeast cells for heat, indicating a specific role of non-degradative K63-linked chains in cells under stress [84]. Recently, K63-ubiquitination was rediscovered in this regard, when it was shown that lack of K63 ubiquitin diminishes the cellular resistance to oxidative stress [85]. The authors identified several protein groups as K63-ubiqutination targets, the largest group associated with translation. The effect of ubiquitination on these proteins, however, remains entirely unknown and will be of interest to investigate in the future. Considering that oxidative stress is an integral part of the ischemic cascade, this regulation could undoubtedly be also important in neurons exposed to ischemia.

Ubiquitin and Cerebral Ischemia-Reperfusion Injury

Ubiquitination Is Induced in the Brain by Ischemia-Reperfusion

Cerebral ischemia puts an immense stress on cells in the affected brain area, and it seems reasonable that brain cells react to this stress with an increase in ubiquitination. Indeed, it was first shown by Hayashi et al. in 1991 that sublethal forebrain ischemia leads to elevated ubiquitin immunoreactivity as well as appearance of high molecular weight ubiquitin conjugates in the rat hippocampus [86]. In a follow-up study, the same authors reported that ubiquitination was increased in the early reperfusion period after global ischemia in the gerbil cortex and hippocampus with a peak at 30 min to 1 h and tended to be resolved by 12–24 h [87]. A similar pattern of ubiquitination was also observed after focal cerebral ischemia in rat and mouse cortices [88, 89]. In both ischemia models, reperfusion was absolutely required for ubiquitination [87, 89], presumably because ATP, which is essential for ubiquitin conjugation, is limiting in ischemic tissue prior to reperfusion. This has not been experimentally tested yet and needs further assessment. Another possibility would be that reperfusion provides the inducing signal for ubiquitination, e.g., by production of free radicals or the activation of signaling cascades associated with excitotoxicity and beyond. Interestingly, pure hypoxic insults in rats did not increase ubiquitination in the post-hypoxia recovery period [90, 91], possibly indicating that lack and re-establishment of blood flow (and the associated availability of glucose) may be essential for activation of ubiquitination. However, additional research will be required to confirm this, since one study used mild hypoxia (15% oxygen) that may not induce sufficient stress [90], while the other study was performed in immature brains [91].

Prevalence of Post-ischemic Ubiquitination in Neuronal Detergent-Insoluble Fractions

Since neurons appear to be most vulnerable to ischemic stress, they are assumed to be the primary source of increased ubiquitination post-ischemia. Indeed, immunocytochemistry of brain sections after ischemia shows a drastic change in ubiquitin immunoreactivity preferentially in cells with neuronal morphology at different times of reperfusion [88, 92–94]. However, it has not been systematically assessed whether glial cells are also affected, thus warranting further investigation.

Where does elevated ubiquitination occur in neurons? As determined by immunocytochemistry of brain sections, most studies found that both global and focal ischemia leads to a redistribution of ubiquitin from an even staining covering the entire cell to a selected perinuclear and dendritic localization [88, 91, 92, 94, 95] (Fig. 3). One study found ubiquitin in the nucleus of hippocampal pyramidal neurons after ischemia/hypoxia [90]. This discrepancy may be the result of the different ischemia model used, or, more likely, due to staining with different ubiquitin antibodies. In fact, it is well established that dependent on their preferential recognition of diverse chains, free or conjugated ubiquitin, or epitopes in the ubiquitin protein, ubiquitin antibodies show great variability when used in immunohistochemistry (examples in the brain after ischemia include [94, 96]). To avoid this drawback, the localization of ubiquitin after ischemia can also be assessed biochemically using subcellular fractionation. Hayashi et al. performed in 1992 the first fractionation experiment where they showed that ubiquitin elevation is prominent in mitochondrial, synaptic membrane, and microsome fractions, but not in nuclei or cytosol [97] (Fig. 3). They also discovered that ubiquitin conjugates are almost exclusively formed in the Triton X100 (Tx100) insoluble fraction, which

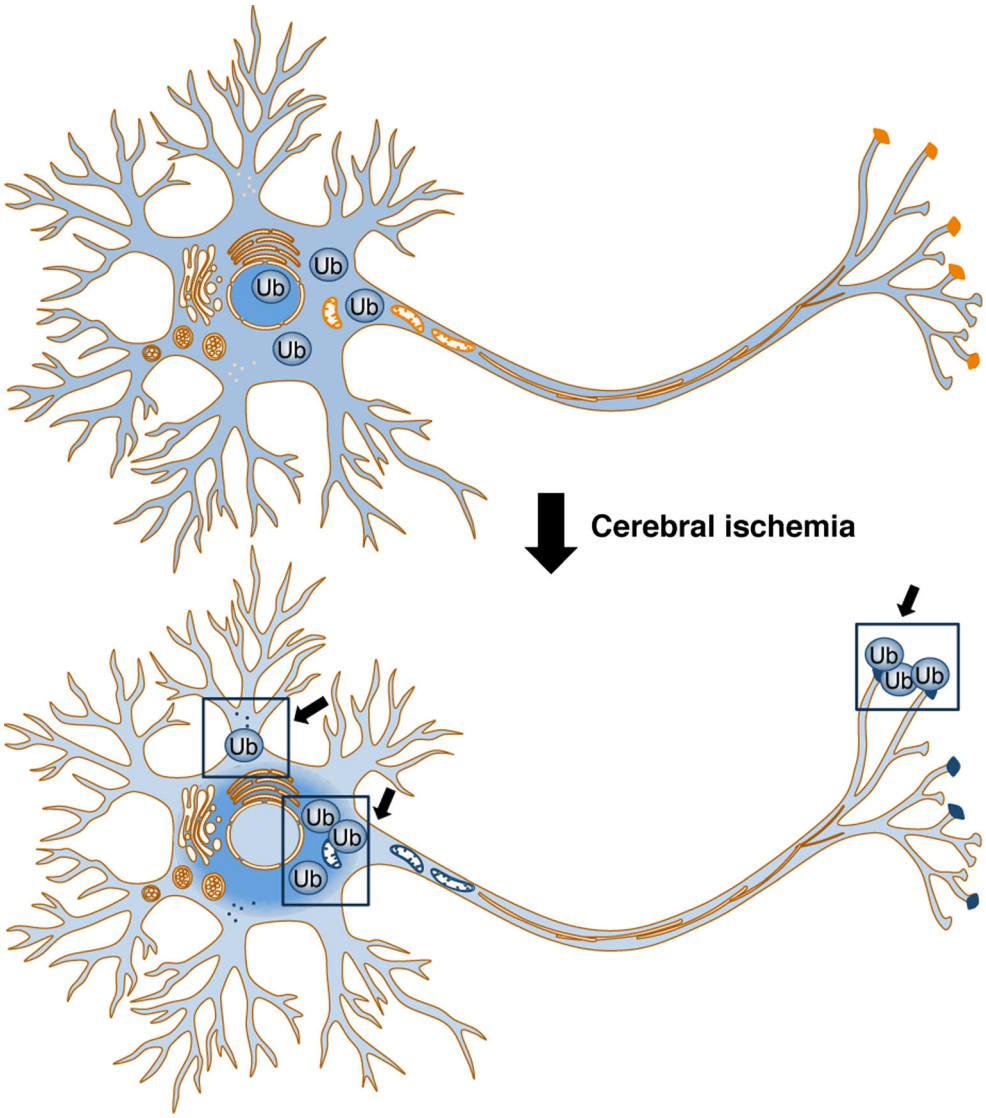

Fig. 3 Cerebral ischemia-reperfusion leads to a redistribution of ubiquitin in neurons. Schematic representation of ubiquitin distribution in pre- and post-ischemic neurons. Under physiological conditions, ubiquitin is evenly distributed throughout the nucleus and cytoplasm showing little to no association with any organelles or microsomal structures (upper panel). After ischemia, ubiquitin is found strongly associated with the postsynape, mitochondria, and microsomes. In addition, redistribution to perinuclear regions is apparent (lower panel). Main accumulation areas of ubiquitin after ischemia-reperfusion are marked with boxes and arrows. Regions with darker blue shades indicate areas containing more ubiquitin. *Ub* ubiquitin

was confirmed by multiple studies in different ischemia models thereafter [88, 89, 98, 99]. It remains unclear to date what this insolubility refers to. On the one hand, the resistance of ubiquitin to high concentrations (up to 2%) of Tx100 could indicate an accumulation of ubiquitin-modified proteins in insoluble aggregates analogous to neurodegenerative diseases. Although this is not entirely impossible, it seems unlikely that such an aggregation would occur on the large scale seen within the short timeframe (minutes) observed after ischemia [89], especially as there is virtually no time when ubiquitin is found in a soluble state.

Alternatively, some intracellular components in neurons are by default largely insoluble and could preferentially be targeted by ubiquitination. One such compartment is the post-synaptic density (PSD), which is only minimally soluble in detergents like Tx100 and only efficiently solubilized with high concentrations of sodium dodecyl sulfate (SDS) or urea. Indeed, it was shown in one report that ubiquitinated proteins are highly enriched in the PSD after ischemia [93]. Supportive of this idea, global post-ischemic ubiquitinated proteins found in the Tx100-insoluble brain fraction by mass spectrometry include many proteins that show a tight connection to the PSD. Among the highly ubiquitinated proteins were CaMKIIα, β, γ and δ, the vesicle-fusing ATPase NSF, PKCβ and γ, NMDA receptor subunits epsilon-1 and 2 (NR2A and NR2B), disk large-associated proteins 1 and 2 (GKAP and DLG2), the calcium-binding protein hippocalcin, Ras GTPase-activating protein (SynGAP), and leucine-rich repeat-containing protein 7 (LRRC7/densin-180) [98]. Although PSD proteins seem to make up much of the ubiquitinated proteome after ischemia, it is entirely unclear what functional consequence this ubiquitination has on

synaptic activity. This important question needs to be addressed in future studies.

Ubiquitination Types Induced by Cerebral Ischemia Are Diverse

In order to predict the impact of ubiquitination on target proteins, it is useful to assess the associated ubiquitin type. As determined by mass spectrometry, prevalent ubiquitin chains found after global forebrain ischemia include K6, 11, 48, and 63 linkages [98] (Fig. 4). Likewise, after focal ischemia, we detected with lysine specific antibodies both K48 and K63 chains [100]. The presence of degradative and non-degradative ubiquitin chains suggests that opposed to popular assumption, proteasomal blockade is likely not the only reason for post-ischemic poly-ubiquitin enrichment. Neither the conditional neuronal knockout of proteasomes in mice [101] nor proteasomal inhibition in yeast [85] is capable of inducing K63 poly-ubiquitination, pointing to alternative mechanisms that contribute to increased ubiquitination levels after ischemia.

Factors Contributing to Elevated Post-ischemic Ubiquitination

Ischemia-Related Changes in Ubiquitin Conjugation

Elevated ubiquitination after ischemia could be caused by an up-regulation of ubiquitin gene expression leading to greater availability of ubiquitin for conjugation. However, as the increase in conjugates is accompanied by the depletion of free ubiquitin [88, 89, 96], the elevated ubiquitination is likely not a result of increased ubiquitin synthesis. This hypothesis is confirmed by the differential expression analysis of ubiquitin genes before and after transient forebrain ischemia in rats, which shows that Uba levels remain entirely unchanged and production of Ubb and Ubc is not activated earlier than 4–6 h into reperfusion [102]. Such increased gene expression occurs too late to account for the increased post-ischemic ubiquitination (Fig. 4).

It is reasonable to hypothesize that augmented activity of certain ubiquitination enzymes is responsible for the vast increase in ubiquitin conjugation after stroke. However, owing to the complexity of the ubiquitination system, this hypothesis has not been tested experimentally. As mentioned before, up to 25,000 different enzyme combinations can mediate target ubiquitination, all or only one of which could potentially be involved in ubiquitination regulation after ischemia. Proteome-wide screens with an easy readout will have to be created to address this issue in the future. In the meantime with the identification of specific ubiquitination targets, a more biased approach can be undertaken to study potentially involved ligases. For example, ubiquitin ligases for some NMDAR subunits (Nedd4–1 and Fbx2) and GKAP (Trim3) under physiological conditions have been identified and could be examined for their ubiquitination capacity after ischemia [103–105]. Another option is to consider ubiquitin ligases that are classically involved in stress regulation and neuronal degeneration. In this regard, the increased activity and level of a few specific ubiquitin ligases have been associated with neuroprotection after cerebral ischemia (Fig. 4). CHIP, an ubiquitin ligase that controls chaperone activity and levels during stress, prevents oxygen/glucose deprivation (OGD)-induced neuronal death when it is overexpressed in vitro in hippocampal slices and in vivo in rats after global ischemia [106]. CHIP overexpression also leads to a reduction in ubiquitinated proteins after OGD; however, this could be the result of decreased neuronal injury. The ubiquitin ligases Nedd4–2 and Itch are induced by focal ischemia in rats in surviving neurons, suggesting that they may be associated with neuroprotection [107]. Also, the Nedd4 adaptor protein Ndfip1 is protective in stroke, since knockout mice exhibit larger infarcts [108]. Since up-regulation of these proteins was measured beginning at 12 h of reperfusion, and maximal regulation was detected at 24 to 48 h post-ischemia, the relevance for early ubiquitination after ischemia is elusive. Another ubiquitin ligase with altered protein level after ischemia is Parkin. Both OGD in neuroblastoma cells as well as focal ischemia in mice was reported to essentially reduce Parkin levels, which leads to mitochondrial fragmentation and potentially ER stress [109, 110]. Like the other ubiquitin ligases, also Parkin protein is neuroprotective, but again, how Parkin levels relate to early ubiquitination after ischemia is not known. In summary, the elevated ubiquitin levels after ischemia cannot be attributed to an increase in ubiquitin protein levels, but are likely caused by an up-regulation of components of the ubiquitin conjugation machinery, the identity of which is entirely unknown to date (Fig. 4).

Ischemia-Related Changes in Ubiquitin De-conjugation

No deubiquitinase (DUB) has been studied for a specific involvement in elevated ubiquitination after stroke, but the activity of two DUBs has been linked to the ischemic outcome (Fig. 4). UCH-L1 is a DUB that is highly expressed in neurons [111] and has gained attention for its protective role in Parkinson's and Alzheimer's disease [112, 113]. Analogous to neurodegenerative diseases, overexpression of UCH-L1 protects neurons from hypoxic injury [114] and down-regulation of UCH-L1 activity in neuroblastoma cells increases OGD-induced cell death [115]. In neurodegeneration, the protective effect of UCH-L1 has been mostly attributed to its potential to prevent Aβ and α-synuclein aggregation by promoting APP and α-synuclein degradation [112, 113]. UCH-L1 potently recycles and stabilizes free ubiquitin

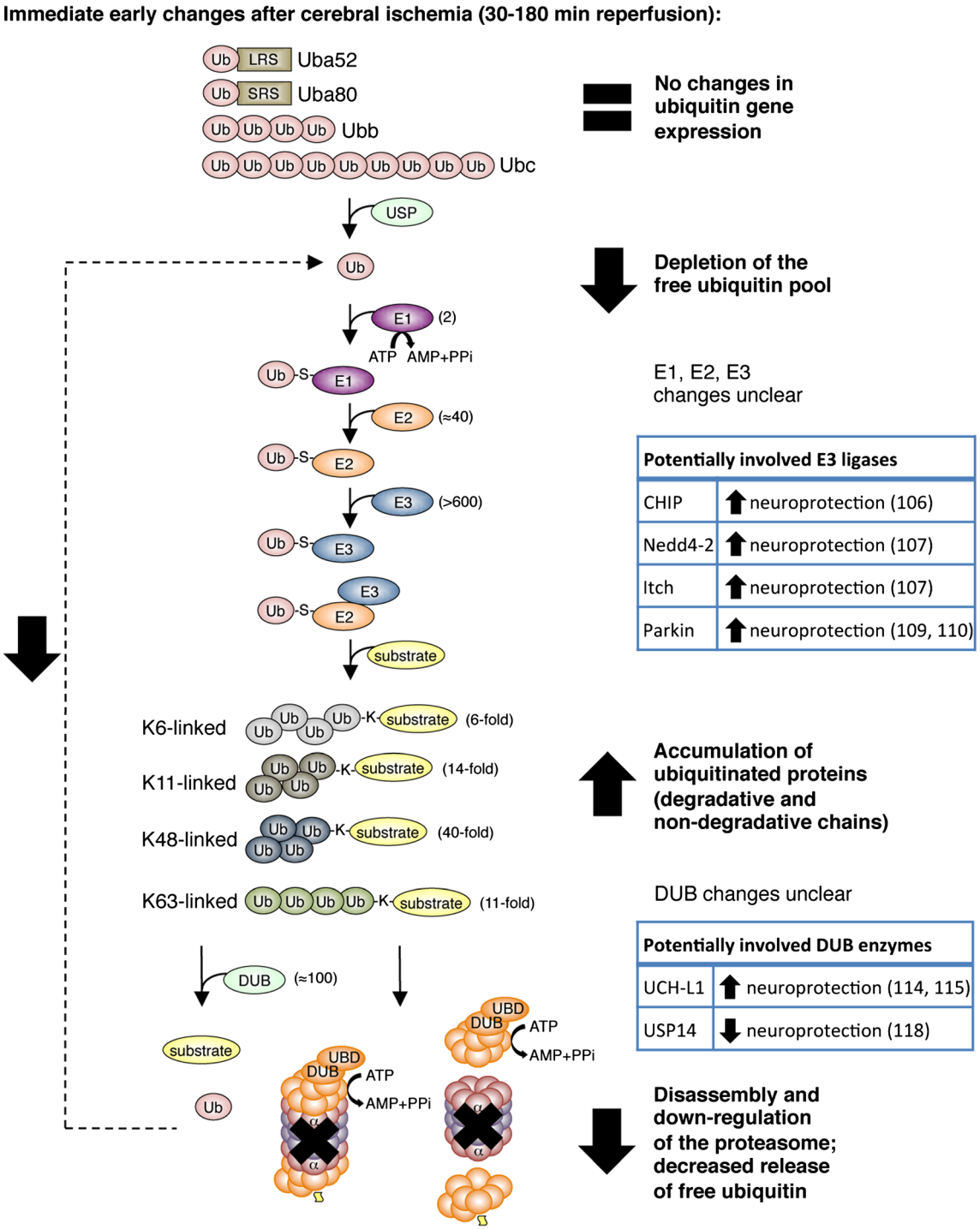

Fig. 4 Post-ischemic changes in the ubiquitin-proteasome system in early reperfusion. A 6-, 14-, 40-, and 11-fold induction in K6-, K11-, K48-, and K63-linked ubiquitinated substrates that is accompanied by decreased free ubiquitin has been found 30–180 min after ischemia. While ubiquitin gene synthesis remains unaffected early after ischemia, there are some reported changes in the ubiquitin conjugation, de-conjugation, and degradation systems that have not been well defined yet and are summarized in this figure. The increased ubiquitination after ischemia is likely a consequence of these converging mechanisms. Known up- or down-regulation of ubiquitin-proteasome components is marked with upward- and downward-facing arrows. *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *DUB* deubiquitinase, *E1* ubiquitin-activating enzyme, *E2* ubiquitin-conjugating enzyme, *E3* ubiquitin-ligating enzyme, *K* lysine residue, *LRS* large ribosomal subunit, *PPi* pyrophosphate, *S* thiolester, *SRS* small ribosomal subunit, *Ub* ubiquitin, *UBD* ubiquitin-binding domain, *USP* ubiquitin-specific protease

derived from proteasomal and non-proteasomal sources and contains a DUB-independent ubiquitin ligase activity, both of which have been shown to contribute to the efficient degradation of disease proteins [113, 116, 117]. In ischemia, the mechanism of protection has not been elucidated. However, it was shown that after hypoxia pharmacological inhibition of UCH-L1 for 24 h increases ubiquitinated proteins in primary neurons [114], which could contribute to hypoxic injury, although direct evidence for this is lacking. In any case, earlier time points need to be studied to clarify the impact of UCH-L1 on the immediate increase in ubiquitination after ischemia. Another DUB with a probable role in stroke and neurodegeneration is USP14, which negatively regulates the proteasomal degradation of ubiquitinated proteins by trimming K48 ubiquitin chains [118]. Inhibition of USP14 was shown to be protective in Alzheimer's disease cell models by facilitating Tau degradation through enhancing proteasomal activity [119]. Injection of a specific inhibitor of USP14 (IU1) [118] attenuated ischemic neuronal injury in a focal ischemia mouse model [120]. The protection correlated with an enhancement of proteasomal activity and reduction of ubiquitinated proteins measured 24 h after ischemia [120]. It is not clear if the decrease in ubiquitinated proteins is neuroprotective per se, or only a measure of less injury at this time-point. As with UCH-L1, more studies looking at earlier times of reperfusion will be necessary to causally connect USP14 with ubiquitination after ischemia.

Ischemia-Related Changes in Ubiquitin Conjugate Clearance

The Proteasome

Augmented presence of ubiquitination could be caused by a blockade in degradation of modified proteins, as one would observe with the use of proteasome inhibitors. The proteasome has been extensively studied after cerebral ischemia, and the consensus is that classical 26S proteasomal activity is indeed reduced during ischemia and also in the reperfusion period, where it recovers gradually (Fig. 4) [89, 121–127]. The mechanism of down-regulation of proteasomal activity after ischemia is not clear, especially whether it is actively repressed, or the repression is a consequence of ischemic damage. It should be noted here that proteasomal activity was mainly measured by its ability to cleave fluorogenic tri- or tetrapeptides containing consensus cleavage sites in vitro. Since these peptides are small enough to enter the proteasomal 20S core regardless of 19S presence, they are only suitable for measuring catalytic core activity, and limited conclusion can be drawn regarding ATP requirements and necessity for a complete 26S assembly [128]. For example, it was shown that 19S and 20S particles partly separate after ischemia [123]; however, the mode of measurement does not capture this change. In fact, oxidative stress was repeatedly reported to result in the dissociation of 20S and 19S subunits in other models, which was protective and necessary for the 20S proteasomes to clear oxidized proteins [30, 129]. In addition, calpain cleavage of the Rpn10 19S subunit was observed in cultured neurons with mitochondrial stress, leading to proteasome inactivation [130], which could also be relevant after ischemia.

There is some evidence that proteasome subunits are targeted by ROS after ischemia that may inhibit its activity. Proteasomal subunits persistently stain positive for HNE and co-immunoprecipitate with DNP from 1 to 24 h after focal ischemia [125, 126]. The same was observed after myocardical infarction in the heart [131]. Whether the modification with ROS is causative for proteasomal inhibition is not clarified. First, proteasomal inhibition seems to precede ROS production, and ROS modification of the proteasome persists even when activity is recovering [89, 121, 125, 126]. Second, at least in the heart, proteasomal activity is extremely resistant to ROS levels, and mapping of oxidized subunits detected a predominant modification of α, not β subunits that actually carry the catalytic activities [132]. Modified proteasomal subunits are still to be identified in the ischemic brain.

Regardless of how proteasomal core activity is reduced after ischemia, does this cause the accumulation of ubiquitinated proteins? On the one hand, inhibition of the proteasome is detected at early reperfusion times before ubiquitin accumulation starts after ischemia; therefore, it is possible. On the other hand, detected K63 ubiquitin chains after ischemia [98, 100] imply an alternative mechanism for accumulation, at least in part. Preservation of proteasomal activity after stroke is often linked with a reduction in ubiquitinated proteins. For instance, protein ubiquitination after transient global ischemia was reduced by pretreatment of rats with trehalose that maintained post-ischemic proteasomal activity [133]. Likewise, USP14 inhibition enhanced proteasomal activity after stroke and led to less ubiquitination [120]. Mice with transgenic over-expression of Ubqln-1, a proteasomal adaptor protein that enhances proteasomal degradation, showed less accumulation of ubiquitin [134]. However, a causal relationship between proteasomal inhibition and ubiquitination has not been determined and lower levels of ubiquitination might just reflect less insult, which was observed in all studies. More research needs to be carried out to properly address whether enrichment of ubiquitinated proteins after cerebral ischemia is a direct result of proteasomal impairment.

Another interesting question is what the reduced proteasome activity means for stroke outcome. Experimental evidence points to a down-regulation of the UPS in brain ischemia that correlates with neuronal death and incubation of neurons with proteasome inhibitors was shown to induce cell

death. However, inhibition of the proteasome after ischemia is only between 20 and 50%, and it has been shown that a reduction of 80% is necessary to have an effect on cellular homeostasis [135]. Yet, a lower degree of inhibition may impair cell viability during stress conditions, and this needs to be still assessed.

Seemingly opposing a detrimental effect of proteasomal inhibition, proteasome inhibitors were shown to provide neuroprotection in various models of stroke. Intravenous, intraperitoneal, and intrastriatal injections of proteasome inhibitors MLN519, Bortezomib/Velcade, CVT-634, and BSc2118 after ischemia lead to neuroprotection [136–141]. Except for Doeppner et al. in 2012, inhibition of brain proteasomal activity was not verified in these studies and it is unclear whether the inhibitors can cross the blood-brain barrier. It has been suggested that inhibitors likely act via a brain-independent mechanism by attenuating neuroinflammation and blood-brain barrier breakdown after ischemia. In fact, proteasome inhibitors when administered at high concentrations and long durations induce neuronal cell death and do not protect isolated neurons from in vitro ischemia [142, 143].

Autophagy

Alternatively to the proteasome, clearance of ubiquitinated proteins can be executed by autophagy, which is mainly responsible for recycling organelles and large cytoplasmic structures, but may also digest larger ubiquitinated aggregates. Post-ischemic autophagy regulation is poorly studied, and there are conflicting reports on whether autophagy is impaired or activated after cerebral ischemia. One study showed that autophagy is persistently up-regulated in reperfusion after in vivo and in vitro ischemia, and that blockage of autophagy with 3-MA in reperfusion increases infarct volume and cell death [144]. In contrast, in another study, the autophagic flux was decreased after ischemia specifically in neurons destined to die [145]. Whether this leads to ubiquitinated protein accumulation was not specifically assessed. The measurements were carried out from 4-h reperfusion; therefore, it is hard to say whether this could be relevant for the early increase in ubiquitinated proteins.

Ubiquitination After Ischemia—Good or Bad?

Post-ischemic ubiquitination is specifically elevated in brain regions that are susceptible to delayed cell death, as opposed to areas where cells die immediately. Focal ischemia induces ubiquitination particularly in cortical regions that are not directly part of the infarct center, while there is only little ubiquitination found within the infarct core [88, 89]. In global ischemia, ubiquitination is highly increased in the hippocampal CA1 layer and cortex [92] that are selectively vulnerable to ischemic stress. Of note, the elevated ubiquitination is detected transiently in neurons that return to a normal morphology after the insult, whereas the ubiquitin stain remains high in neurons that appear to die [88, 92]. Several treatments were shown to alleviate ubiquitination levels after ischemia. Hypothermia preserved ubiquitin immune reactivity in the CA1 region after global ischemia [146] and diminished initiation of ubiquitination after focal ischemia [94]. Ischemic preconditioning led to a decrease in ubiquitination in the otherwise heavily ubiquitinated CA1 area in early and delayed tolerance in global ischemia models [95, 147]. Ischemic post-conditioning reduced ubiquitination as measured 12–72 h after ischemia [148], earlier time-points were not studied. Administration of geldanamycin, an Hsp70 inducer, prior to OGD blocks ubiquitin redistribution from nucleus to cytoplasm in hippocampal slices after insult [149]. Collectively, these observations suggest that ubiquitination contributes to the delayed neuronal cell death after stroke, which has been commonly proposed. However, the presence of ubiquitination in dying cells may not be causative and only be secondary to the cell death trigger, or may even be part of a compensatory protective mechanism that prevents immediate death of these neurons. The reduced ubiquitination in these models is likely due to milder ischemia that causes less stress in neurons. Ubiquitination is elevated immediately and transiently after onset of reperfusion, before any cell death occurs and is in our hands also apparent after insults that do not cause any damage [89]. Alternatively, ubiquitination may indeed increase with ischemic length as shown in a global ischemia model [150], but that may simply reflect higher stress levels. Interestingly, ubiquitination levels were also increased in the liver of squirrels during hibernation [151]. There it was suggested that during suppression of protein synthesis, which is also observed after ischemia, a shut down of proteolysis is required to maintain pools of key regulatory enzymes. Protein ubiquitination may still go on under these conditions.

Ultimately, the important question of whether post-ischemic ubiquitination is neurotoxic or neuroprotective can only be answered by actively manipulating the ubiquitin system after ischemia. But this is technically hard to achieve due to the essential role of ubiquitination in cell homeostasis and the time-window after ischemia in which that would have to be done. A global reduction in ubiquitination by targeting E1 enzymes seems a possibility, but currently, available inhibitors are suboptimal due to irreversibility. Transient knock down of ubiquitin genes with CRISPR or siRNA techniques may be challenging due to the existence of four ubiquitin genes, and efficacy is not guaranteed since mRNA expression of ubiquitin genes does not change with ischemia [102]. There are too many E2/E3 combinations involved to make any approach of targeting them feasible without knowing more about substrates and activation of specific enzymes after stroke.

The Relationship Between Ubiquitin and Sumo After Ischemia

SUMO, Ubiquitin's Cousin

The small ubiquitin-related modifier (SUMO) has in contrast to ubiquitin no direct role in protein degradation, but regulates various other cellular processes, most notably nuclear transport and organization, ribosomal biogenesis and transcription (reviewed in [152–154]). SUMO, like ubiquitin is attached to lysines in substrate proteins through the consecutive action of E1, E2, and E3 enzymes; however, there are some important differences to note. First, the conjugation system is far less complex with one E1 (AOS1/Uba2), one E2 (Ubc9), and about 15 known E3s that are sometimes even dispensable [155]. This is a major advantage over the ubiquitin system when it comes to manipulation of SUMO conjugation. Second, the substrate recognition is carried out independently of the E3 by the E2 enzyme Ubc9 that recognizes a well-defined consensus motif in about 75% of targets [156, 157]. This simplifies the identification of potential SUMOylation substrates compared to ubiquitin. Third, in contrast to a single ubiquitin protein, there are at least three SUMO proteins in mammalian cells [158, 159]. SUMO2 and 3 are identical except for 3 amino acids at the N-terminus, cannot be functionally distinguished, and are consequently referred to as SUMO2/3. SUMO1 shares about 50% identity with SUMO2/3. Both use the same E1, E2, and certain E3 enzymes and have overlapping and individual substrates [160]. SUMO conjugation, like ubiquitin, is essential to cell survival, as deletion of Ubc9 that is used by both SUMO types is lethal in mice [161]. Notably, SUMO2/3 conjugation is heavily induced by oxidative, hypoxic, osmotic, and heat stress [162, 163], whereas SUMO1 modification is generally not affected, with the exception of hypoxia [164, 165], a regulation that is also relevant to the brain [166].

Although ubiquitin and SUMO use different enzymes for conjugation and de-conjugation, there is an extensive interplay between the modifications. First, SUMO can act as recognition signal for ubiquitin ligases, for example RNF4 and Mip1, which process SUMOylated substrates for ubiquitination and degradation [167, 168]. Consequently, inhibition of the proteasome results in the accumulation of ubiquitin as well as SUMO conjugates [169, 170]. Second, some SUMO targets are also ubiquitinated and vice versa, acting in concert or in opposition in determining substrate fate. For example, under hypoxic conditions, SUMOylation of HIF-1α is required for its ubiquitination by VHL [171]. In contrast, in case of Mdm2 and USP25 substrates, SUMOylation and ubiquitination at the same lysine residue have opposing effects on stability and activity, respectively [172, 173]. Finally, there is increasing evidence of mixed SUMO2/3 and ubiquitin chains with degradative and non-degradative function. On the one hand, heterologous SUMO-ubiquitin chains enhance IκBα degradation to initiate NF-κB activation [174]; on the other hand, such hybrid chains promote DNA repair in case of BRCA1 and RAP80 [175].

SUMO and Ubiquitin Are Related After Ischemia

The role of SUMOylation in hypoxia and ischemia has been reviewed very recently and will not be discussed in detail here [176]. Rather, I will focus on the relationship of SUMO to ubiquitin after ischemia since this could give further clues on how ubiquitination could impact the ischemic outcome. SUMO conjugation predominantly consisting of SUMO2/3 chains is neuroprotective after global and focal transient ischemia [177–182], and there are several indications that post-stroke SUMO and ubiquitin might be connected (Fig. 5). First, ischemia-reperfusion injury increases SUMO conjugation with an identical time course as ubiquitination [100, 183]. SUMO and ubiquitin are not only present at the same time in the same brain areas after stroke. They physically interact. Proteomics revealed that SUMO pull-down precipitates ubiquitin after OGD in neuroblastoma cells [184]. Our lab confirmed these data in a mouse focal ischemia model where we co-precipitated SUMO with ubiquitin and vice versa [100]. This was surprising to us as contrary to SUMO, ubiquitin was mostly found to exit the nucleus after stroke. However, recent data indicate that at least a fraction of ubiquitin localizes to the nucleus after ischemia [183]. Finally, there is some evidence that ubiquitination is dependent on prior SUMO2/3 conjugation [184] and attaches partially to the same substrates after stroke [183]. At this time, it is definitely a stretch to extrapolate the protective findings of post-stroke SUMOylation to ubiquitin; however, it is tempting to speculate that both modifications might act in concert to protect neurons from ischemic damage.

Challenges and Future Directions

The ultimate research goal in the field is certainly to provide information on how the ubiquitin system after stroke can be targeted for therapeutic and drug development. To achieve this, two major questions need to be answered. The first is how is ubiquitination induced by ischemia. It was long assumed that elevated ubiquitination is purely a consequence of proteasomal inhibition; however, recent data on ubiquitin types and timelines of ubiquitination versus proteasome function after ischemia raise doubt about whether this is indeed the case [98, 100, 123]. The view that ubiquitin conjugates serve purely degradative functions is outdated and should be revisited in stroke research as well. The second question is whether post-ischemic ubiquitination is neuroprotective or detrimental. Data collected so far suggest that changed levels

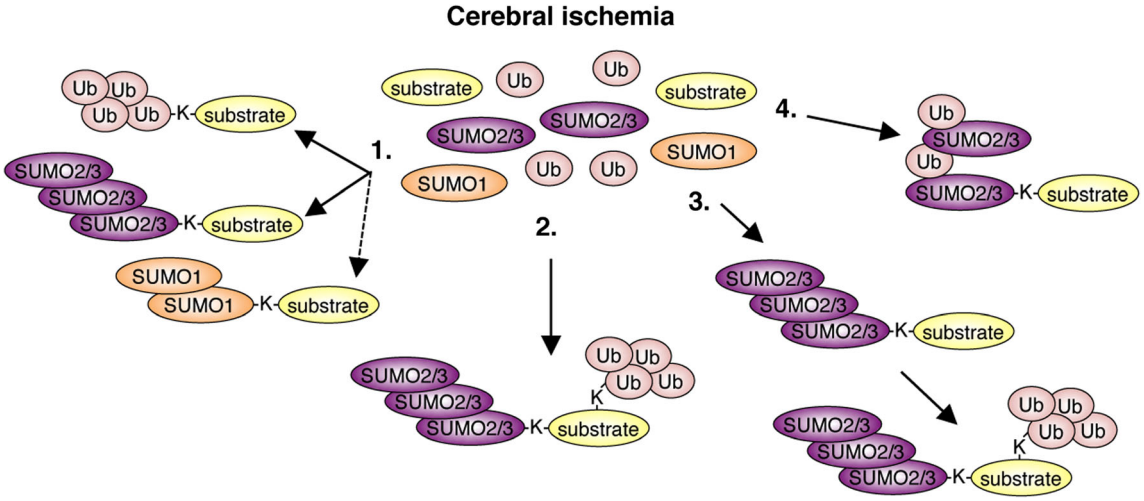

Fig. 5 Ubiquitin and SUMO converge after cerebral ischemia. Several scenarios of how ubiquitin and SUMO interact after ischemia are conceivable and backed up by existing experimental data. First, despite temporal similarities, substrate proteins could be ubiquitinated and SUMOylated independently of each other. The majority of SUMOylated proteins are modified with SUMO2/3, only a minority with SUMO1. In this scenario, the consequences of SUMO and ubiquitin conjugation may be different for individual substrates (pathway 1). Second, ubiquitin and SUMO could be attached to the same substrate, conjointly deciding the substrate's fate. Thereby, SUMO and ubiquitin may be attached simultaneously (pathway 2) or sequentially (pathway 3). Finally, ubiquitin and SUMO2/3 may form mixed chains on certain targets, again affecting a substrates fate together (pathway 4)

of both proteasomal function and/or ubiquitination may influence stroke outcome, thus attesting causality [94, 95, 146–148]. However, all models used outcome-modifying treatments; therefore, an altered ubiquitin-proteasome system may be secondary to alteration of the ischemic damage. Targeted modulation of ubiquitination and proteasomal activity during or after stroke must be carried out to conclusively address causality. Considering that ubiquitin targets many proteins that will be differently regulated, it would be naïve to think that looking at global changes in tissue or cell ubiquitination levels is a good indicator of whether the modification is beneficial or detrimental. Consequently, the path forward would be to identify and characterize ubiquitination substrates and substrate-specific enzymes that are specifically active after stroke. Potential therapeutic approaches could be designed to modulate any of the enzymatic activities central to the ubiquitination system. However, since ubiquitination is essential to cell homeostasis and survival, pharmacological intervention at a broader level would likely be harmful. It would be preferable to target enzymes that confer specificity to the system, like the E2, E3, or DUB enzymes, and with that limit the negative effects of global modulation of ubiquitination. Clinical results for cancer and neurodegenerative therapy support this approach, since the most successful ubiquitin pathway drugs have targeted E3 ligases and DUB enzymes (reviewed in [185]). Another appealing approach might be the targeting of individual substrates themselves, for example by inhibition or promotion of binding to ubiquitination enzymes, depending on outcome prediction.

Conclusions

Post-ischemic protein ubiquitination is rapidly and transiently increased in neurons at the periphery of the ischemic territory, a process dependent on reperfusion. Given the importance of ubiquitination for cell homeostasis and stress responses, the impact of this change for neuronal survival and functioning must be significant. There are precedents for therapies targeting the ubiquitination system in cancer and neurodegeneration, which suggests that such interventions may also be applicable to stroke. However, currently, we know little about how ubiquitination is induced after cerebral ischemia, what enzymes are involved and what is the impact of ubiquitination on neuronal fate and ultimately stroke outcome. A better understanding of the roles of ubiquitin in ischemic stroke is necessary before the exploration of modulating ubiquitination for therapeutic purposes can begin.

Compliance with Ethical Standards

Conflict of Interest The author declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

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