Heat stress modulates the GSK-3β levels and Tau phosphorylation

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Abstract

Alzheimer's disease is a prominent neurological disorder, which leads to progressive dementia. The microtubule-associated protein Tau is been considered as one of the major causes of Alzheimer's disease. Physiologically Tau assists in the stabilization of microtubules, contrary to this the pathological state of Tau results in the formation of neurotoxic tangles of Tau. The posttranslational modifications, such as GSK-3β-mediated Tau phosphorylation results in the generation of Tau pathology. Neuroinflammation generated in Alzheimer's disease, contributes to elevated body temperature. The aim of present work is to study the effect of high temperature on Tau phosphorylation. The neuroblastoma cells were exposed to heat stress for 40 minutes. The immunofluorescence and western blot studies suggested that high temperature increases the levels of GSK-3β in cells. Heat stressed cells was also observed to have elevated levels of phosphorylated Tau. Additionally, heat stressed cells found to have modulated nuclear transport as the level of Ran was reduced. The results of present work suggest~~ed~~ that increased temperature could be considered as a risk factor in Alzheimer's disease as it elevated the GSK-3β levels in cells thus, resulting in increased Tau phosphorylation.

Keywords

Alzheimer's disease, Tau phosphorylation, Heat stress, GSK-3β.

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Introduction

Alzheimer's disease is the neurodegenerative disorder, resulting in symptoms-like memory loss, behavioural impairment and locomotory deficits[1, 2]. Alzheimer's disease is hallmarked by two types of protein aggregates, which are senile plaques composed of amyloid-β peptide and neurofibrillary tangles (NFTs) composed up of microtubule-associated protein Tau[3, 4]. The action of gamma-secretase on amyloid precursor protein (APP) results in the generation of amyloid β-42 peptides, leading to generation of extracellular senile plaques[5]. Tau protein is considered to be a cytoskeletal protein, having role in stabilization of the microtubules[6-8]. In the pathological state Tau detaches from microtubules and lead to the formation of intracellular neurofibrillary tangle NFTs[9, 10]. Tau is a 441 amino acid long natively unfolded protein, the domain organization of Tau comprises of projection domain and microtubule-binding domain[10, 11]. The four repeat region present in microtubule-binding domain is associated with the Tau pathology[12]. The four repeat region is prone to several post-translational modifications including phosphorylation, glycation, glycosylation *etc.* Tau phosphorylation is one of the major cause of pathologic state of Tau[13]. Tau phosphorylation contributes to several defects in cell signalling leading to neurotoxicity[14]. The increased load of Tau phosphorylation leads to generation intracellular ROS[15]. Heavy metal such as mercury and iron are known to modulate Tau phosphorylation[16]. Similarly, abnormal phosphorylation of Tau results in reduced microtubule binding of Tau[17]. Thus, phospho-Tau and the Tau specific kinases are considered as a prime target for therapeutic studies in AD. GSK-3β and CDK5 are the principle Ser/Thr kinases, while Src family kinases (SFKs), FYN and the ABL family kinases phosphorylate tyrosine[18]. Several compound such as leptin, which downregulates Tau phosphorylation are designated as potent molecules against AD[19]. Similarly metal ions as Lithium also showed promising results in reducing Tau phosphorylation by inhibiting GSK-3β[20]. GSK-3β and CDK5 phosphorylates Tau on various sites which ultimately results in Tau pathology[21]. Additionally, these kinases are also reported to phosphorylate the GPCR proteins, which contributes to AD pathology[22]. GSK-3β gets phosphorylated at Ser-9 leading to generation of its inactive state pGSK3-β[23]. Several studies have suggested increased levels of GSK-3β in AD condition. Fluctuation of body temperature has been observed in AD patients. Hypothermia has also been marked as a risk factor for AD. The published studies have suggested that hypothermia might down regulate the GSK-3β expression[24]. On contrary the reports suggested that as a result of neuroinflammation AD patient may suffer increased body temperature[25]. The deregulation of nucleocytoplasmic transport is one of the consequences of neurodegeneration. Reduced levels of Ran has been observed in case of neurodegeneration[26]. Similarly the deformities in the arrangement of nuclear pore complex (Nups) also studied in neurodegenerative disease[27]. The aim of our work is to study the effect of increased temperature on GSK-3β levels and Tau phosphorylation. The work was intended to investigate the fact weather increased body temperature could be one of risk factors for AD patients.

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Results

Heat Stress increases GSK-3β levels in cells

GSK-3β is the Ser/Thr kinase, which targets microtubule-associated protein Tau. The increased phosphorylation of Tau leads to aggregation. Thus, GSK-3βis considered to be associated with Alzheimer's disease. Neuroinflammation results in elevated body temperature in Alzheimer's disease (AD)[28]. Here we observed the effect of high temperature on GSK-3β levels in neuronal cells. Earlier studies have suggested that at temperature of 43°C the cells experiences the heat stress leading to generation of several stress responses[29]. Thus in our studies, the cells were incubated at 43°C for 40 minutes to induce the heat stress (Figure 1A). The immunofluorescence studies suggested that heat stress increases the GSK-3βlevels in cells. The elevated GSK-3β indicated that heat stress may contribute to increased levels of Tau phosphorylation (Figure 1B). The quantification of immunofluorescence images supported the results that intensity of GSK-3β increased after exposure

Figure 1

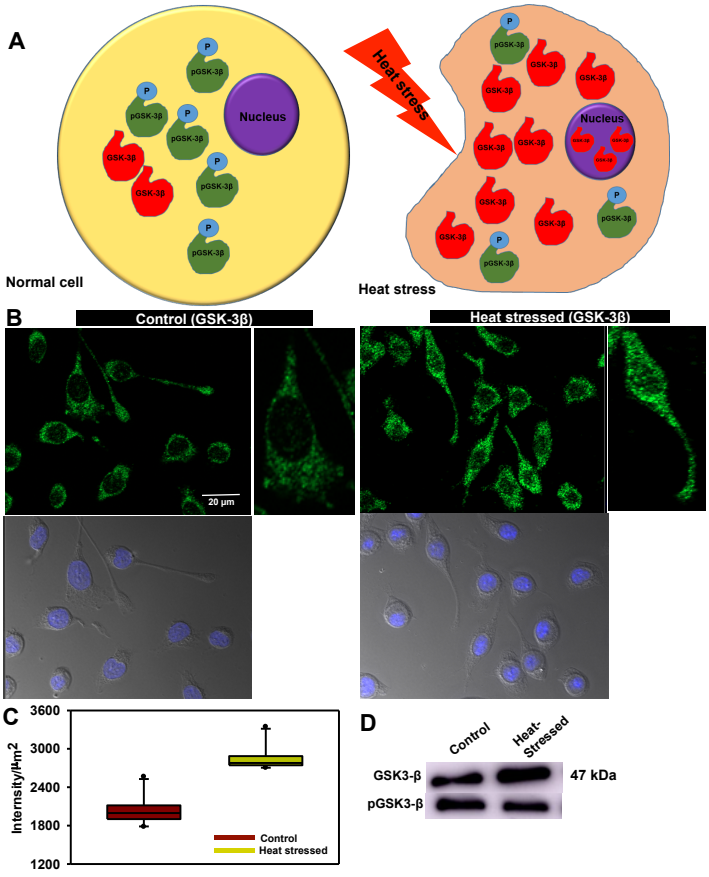

Figure 1. Heat stress increases the GSk-3β expression in cells. A) The diagrammatic hypothesis explaining the effect of heat stress in cells. B) The immunoblot suggested that heat stress at 43°C for 40 minutes increases the expression of GSK-3β in cells. C) The quantification of immunofluorescence images showing the increased

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intensity of GSK-3β in heat stressed cells. D) The immunoblot suggesting the increased levels of GSK-3β in heat stress cells, whereas pGSK-3β levels remains unaltered as compared to control.

to high temperature. The western blot analysis of heat stressed cells suggested that the levels of GSK-3β elevated after heat stress, whereas no changes in pGSK-3β levels were observed. Thus, the above results indicated that heat stress might elevate the levels of GSK3-β, which could be reason for induction of Tau pathology.

Figure 2

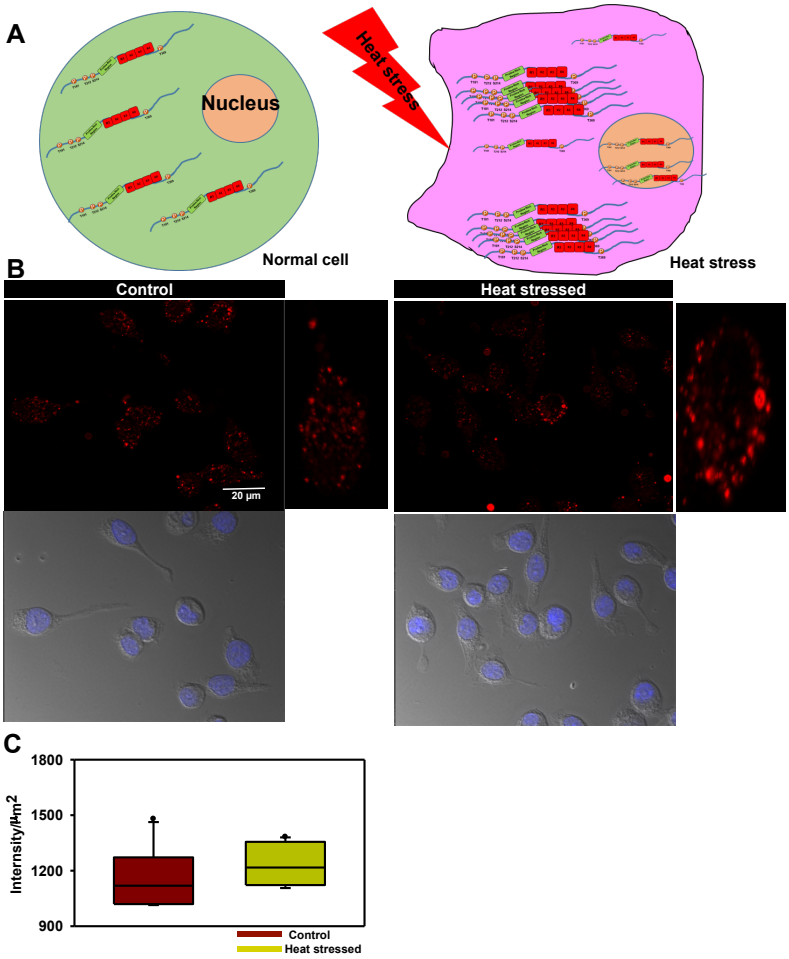

Figure 2. Heat stress increases the phospho-Tau in cells. A) The diagrammatic explanation of effect of heat stress on level of phospho-Tau in cell. B) The immunofluorescence images sowing the presence of phospho-Tau (AT100) in heat stressed cell as compared to control cells. C) The quantification of immunofluorescence images indicating increased intensity of AT100 in heat stressed cells as compared to control cells.

Heat Stress elevates phospho-Tau levels in cells

Several factors contribute to the generation of Tau pathology, among these phosphorylation of Tau is considered as leading cause of Tau pathology. Tau protein has 85 sites which are targeted by various kinases for of phosphorylation. GSK-3β, which phosphorylates Tau, at sites such as S199, S202,

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T231, S235, S396, S413 etc., resulting in its pathological state. In this study we observed the effect of increased temperature on levels of phospho-Tau in cells (Figure 2A). The immunofluorescence images suggested that the cells incubated at 43°C for 40 minutes showed increased levels of phospho-Tau (Ser212/214) (Figure 2B). The quantification of immunofluorescence images supported the fact that intensity of phospho-Tau was elevated in heat stressed cells (Figure 2C). The elevated levels of phospho-Tau in heat stressed cells indicated that increase in temperature could be one of the risk factors for Tauopathy.

Figure 3

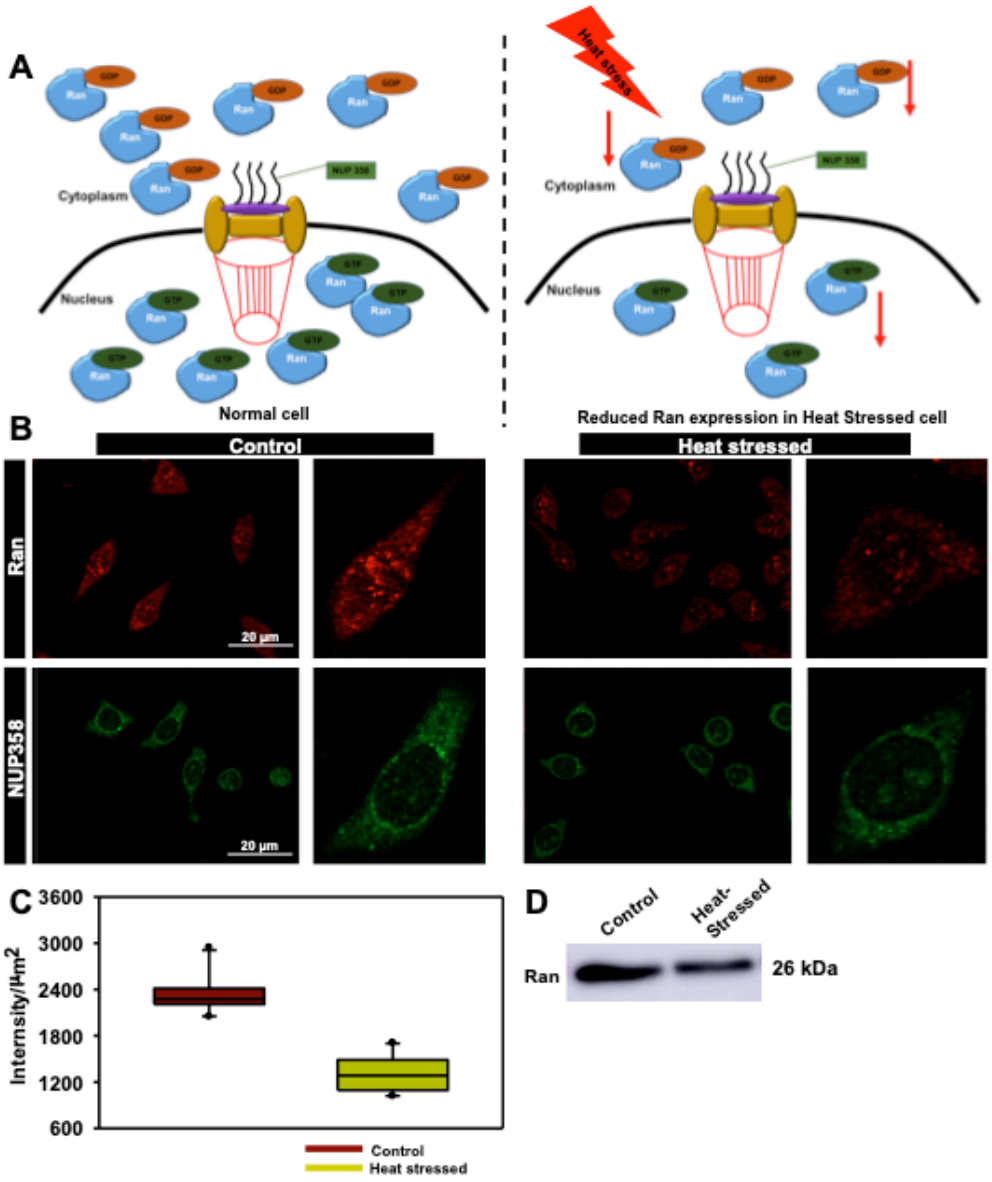

Figure 3. Heat stress modulates nuclear transport. A) The diagrammatic representation of nuclear transport of normal cells and heat stressed cells. B) The immunofluorescence images showing the effect of heat stress on nuclear transport. Ran gradient was reduced in the heat stress cells, whereas the NUP358 morphology was

unaltered. C) The quantification of immunofluorescence images suggests the reduced level of Ran in heat stress cells. D) The immuno blot analysis of heat stressed cells *vs*. control cells. The heat stress cells was observed to have low level of Ran as compared to control.

The effect of high temperature on nucleocytoplasmic transport

The nucleocytoplasmic transport in cells is govern by several carrier proteins. Ras-associated nuclear protein (Ran) is the key protein for nucleocytoplasmic transport. Recent studies suggested that under neurodegenerative condition the Ran gradient gets disturbed, which results in neuronal death[30]. The objective of our study was to observe the effect of high temperature on nucleocytoplasmic transport. (Figure 3A). The immunofluorescence images indicated that the levels of Ran were reduced in heat stressed cells. The modulation in Ran levels could be due to disturbed nuclear transport (Figure 3B). The quantification results suggested that the intensity of Ran lowers down in the heat stress cells (Figure 3C). The immunoblot analysis suggested that the level of Ran protein were decreased in heat stressed cells (Figure 3D). On contrary, we observed no modulation in NUP358 arrangement in heat stressed cells. Thus, these studies evidenced that elevated temperature could modulate the nucleocytoplasmic transport, resulting in the generation of neurodegeneration.

Discussion

The neurodegeneration includes diseases such as include Alzheimer's disease, fronto-temporal dementia, Parkinson's disease, vascular dementia, lewy body dementia, Posterior Cortical Atrophy, Creutzfeldt-Jakob Disease *etc*[31-33]. The protein aggregates of amyloid-β-42 and the microtubule-associated protein Tau are considered as the major cause of Alzheimer's disease[6, 13]. AD is symptomized by a spectrum of symptoms including impaired cognition, memory deprivation, emotional imbalance and problem in performing routine activities[9]. Under the pathological condition, the hyperphosphorylation of Tau leads to the generation of several structural and functional modifications resulting in neurotoxicity [34]. Cells have various classes of kinases, which include serine/threonine kinase and tyrosine kinase [34, 35]. GSK-3β and CDK5 prominent Tau targeting kinases Tau[36], which are considered to be involved in generation of Tau pathology[37]. Several studies claim the involvement of GSK-3β in AD pathology, which includes memory impairment, locomotry dysfunction and behavioural impairments[38]. The amyloid-β-induced neurotic damage have been considered to be mediated by GSK-3β activation[39]. Similarly, increased levels of GSK-3β was observe in the brain of AD patients[40]. Recent studies suggested that the silencing of GSK-3β reduces the tangles formation in mouse model of AD[41]. The selective GSK-3β inhibitor SAR502250 was reported to reduce the behavioural impairment and neuropsychiatric symptoms in rodent AD models[42]. GSK-3β is leads to apoptotic cell death by inhibiting PI3K pathways[43]. In addition to AD, GSK-3β is studied to be involved in other Tau related-frontotemporal dementia[38]. GSK3-β, also

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induces the caspase activity in endoplasmic reticulum stressed cells[44]. Additionally, GSK-3β modulate the Nrf2 signalling pathway under the stress conditions[45]. During bacterial infection GSK-3β modulated the NF- κB levels leading to inflammation[46]. As GSK-3β is reported to be involved in AD pathology, several strategies have been applied to inhibit the GSK-3β. Indirubins and derivatives showed potent inhibition of GSK-3β[47]. Similarly erroneous, alsterpaullone, purvalanol,

Figure 4

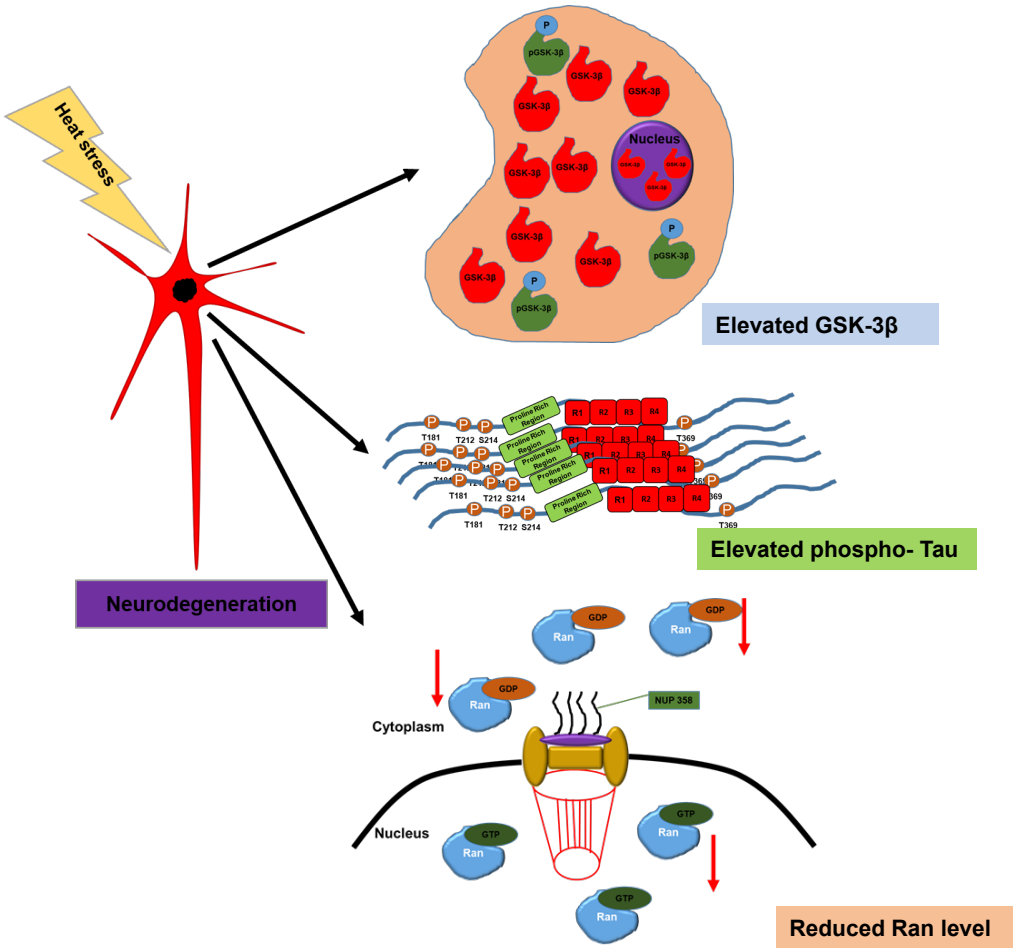

Figure 4. The diagrammatic representation of our hypothesis which suggests that heat stress modulate the GSK-3β levels in cells. The increased GSK-3β in cells contributes to the elevated load of phospho-Tau, which leads to various cell toxicity. Heat stress was found to reduce the Ran level in cells whereas Nup358 was observed to be altered.

pyrazolopyrimidine are also the compounds studied to have effect in downregulation of GSK-3β[48]. The hypothermia in patient with neurological disorders have been reported to modulate the GSK-3β levels[24, 49]. The AD patients also suffer the increased core body temperature as a result of activated inflammatory pathways[25]. The effect of high temperature on GSK-3β levels were not studied yet. Here we observed that heat stress increased the GSK-3β expression in cells, which ultimately led to

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increased phospho-Tau levels in neurons. Ran is one of the key protein of nuclear transport[50]. Phospho-Tau has also been reported to disrupt the nuclear transport by reducing the Ran gradient in cells[30]. In our study we have also monitored that heat stress results in reduction of Ran levels in cells indicating the impaired nucleocytoplasmic transport. Thus, we suggest that heat stress could act as risk factor in AD which ultimately led to Tau hyperphosphorylation.

Conclusion

Tau phosphorylation is considered as a major cause of Tau pathology. The GSK-3β is associated with Tau phosphorylation. The results of present work suggest us that high temperature could increase the expression of GSK-3β and phospho-Tau in neurons. Additionally, heat stress modulated the nuclear transport by the reducing level of Ran although the Nup358 arrangement and nuclear morphology was observed to be unaltered. Thus, the overall results suggest that high temperature could contribute to AD pathology and hence, it could be considered as a risk factor for AD.

Materials and Methods

Chemicals and Reagents

Triton X-100 was purchased from Sigma. DMEM advanced F12 media (12634010), fetal bovine serum (1600004), penstrep cocktail (10378016), anti-fungal agent Anti-anti (15240062) were purchased from Gibco. GSK-3β monoclonal antibody, AT100 (MN1060), Goat anti-mouse Alexa fluor plus 555 (A32727), Goat anti-rabbit Alexa IgG fluor 488 (A-11008) antibodies were purchased from Thermo fisher. Ran (ab11693) antibody was purchased from Abcam. The secondary antibodies Goat anti-Rabbit HRP conjugated and Goat anti-mouse HRP conjugated were purchased from Thermo fisher, Clarity™ Western ECL Substrate (1705060 - Bio-Rad).

Immunoblot assay

The mouse neuroblastoma cells Neuro2a (ATCC CCL-131) were acquired from ATCC. The cells were maintained in advanced DMEM/F-12 media supplemented with 10% fetal bovine serum. The cells were kept in incubator at 37°C supplied with 5% CO_2. The cells were regularly passaged and maintained.

The effect of heat stress were studied in mouse neuroblastoma cells (Neuro2a cells). For the western blot analysis neuronal cells were seeded at density of $1.5X10^5$ cells/well in 12 well poly-lysine coated plate. The cells were subjected to heat stress at 43°C for 40 minutes. The cells were lysed with RIPA buffer supplemented with protease inhibitor cocktail. The lysate was centrifuged at 12000 rpm for 20

minutes at 4°C and the supernatant was collected and loaded on 10% SDS-PAGE. The protein was transferred to PVDF membrane using semi-dry blot transfer unit (GE). The blots were incubated with 5% skimmed milk for 60 minutes at room temperature for blocking the nonspecific sites. Following blocking, the blots were incubated with specific primary antibodies *viz*. GSK-3β(1:1000), pGSK-3β(1:1000), AT100 (1:1000), Ran (1:10,000). The blots were incubated with primary antibody at 4°C for overnight. The blots were washed thrice with PBST, pH 7.4 and incubated with HRP-conjugated secondary antibody for 1 hour at room temperature. The blots were washed trice with PBST and developed using clarity max® Bio-Rad ECL solution. The blots were imaged in Amersham AI600 chemiluminescent imager. Each set of experiment were repeated twice and the quantification was done using ImageJ software.

Immunofluorescence

The immunofluorescence studies were further carried out to validate the effect of heat stress on various cellular signalling. Neuro2a cells (25,000 cells/well) were divided into two groups, group one comprised of cells exposed to heat stress and second group cells were kept at 37°C, which was designated as cell control. After the incubation, the cells were washed with 1X PBS, followed by 4% paraformaldehyde to fix the cells. The cells were permeabilized using 0.2% Triton X-100 followed by blocking with 2% horse serum for 60 min. The cells were incubated overnight with monoclonal primary antibodies GSK-3β (1:100), AT100 (1:100), Ran (1:1000), NUP 358 (1:1000) at 4°C. After three subsequent washes of 1X PBS, the cells were incubated with secondary antibodies goat anti-rabbit Alexa 488 (A11034) and goat anti-mouse Alexa 555 (A32727) for 60 minutes at 37°C. After incubation the cells were washed with 1X PBS and 300 nM of DAPI was added onto cells. After the final wash with 1X PBS, the coverslips were mounted on slides (blue star) and sealed. The samples were imaged on Zeiss Axio Observer 7 with Apotome 2.0. The mages were quantified in Zen 2 (Blue) software and the statistics were plotted using SigmaPlot 10.0.

Statistical analysis

The statistical data for the fluorescence measurement or viability assay was plotted by using either duplicate or triplicate reading. Untransformed (raw) data were analysed and plotted by SigmaPlot 10.0 software. The data was analysed for the significance by Student's *t*-test.

Competing Interest

The authors declare that they have no competing interest.

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Author Contributions

S.C, T.D designed and carried out the experiments. T.D and S.C analyzed the data. S.C. conceived the idea of the project, provided resources, supervised and wrote the manuscript. All authors contributed to the discussions and manuscript review.

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