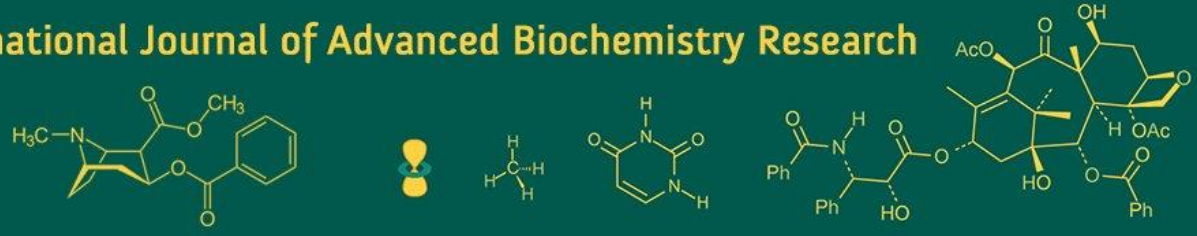
International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(12): 547-551
www.biochemjournal.com
Received: 15-10-2025
Accepted: 18-11-2025

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Rab GTPase dysregulation as a marker of Tau-mediated disturbance in Endosomal dynamics in Neuro2A cells

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i12g.6559

Abstract
Rab GTPases are central regulators of vesicle trafficking, and their dysregulation is increasingly linked to the pathogenesis of Alzheimer's disease (AD). To understand how tau-mediated cellular stress affects endosomal dynamics, we employed an okadaic acid (OA)-induced tau hyperphosphorylation model in Neuro2A cells. Cells were treated with OA (100 nM, 4 h), and the expression of Rab5, Rab7, Rab9, and Rab11 was assessed by Western blotting followed by densitometric analysis. OA-induced tau hyperphosphorylation resulted in a marked reduction of Rab5, an essential early endosomal marker, indicating impaired early vesicle sorting. Rab7, which mediates the maturation of Rab5-positive endosomes into late endosomes, was also decreased, suggesting a disruption in endosomal progression. In contrast, Rab9, a late endosomal marker associated with trafficking to the trans-Golgi network, showed elevated expression upon OA exposure, consistent with a shift toward late endosomal accumulation. Rab11, which governs recycling endosome function, exhibited altered expression patterns indicative of impaired recycling pathways under tau-driven stress. Together, these findings suggest that tau hyperphosphorylation perturbs the balance of early, late, and recycling endosomal trafficking, potentially contributing to vesicular dysfunction observed in AD. Further mechanistic studies are needed to elucidate how Rab dysregulation integrates into tau-mediated neurodegenerative pathways.

Keywords: Okadaic acid, Rab 5, Rab 7, Rab 9, Rab 11, Neuron

1. Introduction
Alzheimer's disease (AD) is characterized not only by hallmark amyloid-β plaques and neurofibrillary tau tangles but also by profound disturbances in intracellular vesicle trafficking [1]. Emerging evidence shows that defects in endocytosis and endosomal maturation occur early in AD pathogenesis and may contribute to synaptic dysfunction, altered receptor turnover, impaired autophagy-lysosomal degradation, and the propagation of pathological tau [2-5]. Endocytosis plays a central role in neuronal physiology by enabling the uptake of extracellular molecules, membrane proteins, and lipids, directing these towards recycling, retrograde transport to the Golgi, or degradation via lysosomes [6, 7]. These tightly regulated trafficking pathways depend on coordinated interactions between motor proteins, cytoskeletal elements, and vesicles [8-10].

Rab GTPases act as master regulators of these pathways by specifying vesicle identity, movement, docking, and fusion [11]. Localized to distinct endosomal compartments, Rab proteins orchestrate a sequential transition from early to late endosomes and further determine cargo recycling or degradation [12]. Rab5 governs early endosome formation and maturation, Rab7 facilitates the conversion of early to late endosomes and Rab9a and Rab11 coordinate recycling routes to the trans-Golgi network and plasma membrane [9, 11, 13, 14]. Given the critical roles of these Rabs, disruptions in Rab GTPase expression or function can profoundly affect neuronal homeostasis and are increasingly implicated in AD-related vesicular pathology.

Tau hyperphosphorylation—an early event in AD—has been shown to impair vesicle trafficking machinery [15]. Okadaic acid (OA), a potent PP2A inhibitor, is widely used to model tau-mediated cellular stress [16]. Understanding how tau hyperphosphorylation alters Rab-mediated endosomal dynamics may provide insights into the vesicular disturbances underlying AD progression.

The aim of the present study was to investigate how tau-mediated cellular stress influences endosomal maturation and vesicular trafficking by evaluating the expression patterns of key Rab GTPases in an okadaic acid-induced Alzheimer's disease model using Neuro2A cells.

2. Materials and Methods

2.1 Cell culture

Neuro 2A cells, *Mus musculus* neuroblastoma cells (a kind gift of Dr. A Jayakumaran, Emeritus Professor, Department of Biotechnoogy, University of Kerala and Dr. Jackson James, Scientist, RGCB, Thiruvananthapuram) were cultured in Dulbecco's modified Eagles medium (DMEM) (Cat. No: 12100-046, Gibco, NY, USA) with 10% Fetal bovine serum (FBS) (Cat. No: 10270-106, Gibco, NY, USA), 1% antibiotic (HIMEDIA Cat.no: A001, MH, India) and 2 mM Alanine-Glutamine (Cat.no: G8541 Sigma-Aldrich, USA) and maintained at 37 ºC in the presence of 5% CO_2 and 95% humidity.

For okadaic acid challenge, Neuro 2A cells were grown until confluency of 80-85 %. The cells were then shifted to low serum conditions (0.5% FBS) and incubated for 30 minutes at 37 °C. The cells were then challenged with 100 nM okadaic acid (Cat. No: 5934, Cell Signaling Technologies, Danvers, MA) and incubated for 4 hours. Following treatment, the cellular morphology was observed under a light microscope (EVOS Floid Cell Imaging station, Invitrogen, USA).

2.1 Cell lysate preparation, protein isolation and estimation

After incubation, cells were washed with ice cold 1 X phosphate buffered saline (pH 7.4). RIPA lysis buffer (Cat. No. 20-188, EMD Millipore Corp, MA, USA) containing protease phosphatase inhibitor (Cat. No. PPC1010, Sigma Aldrich, USA) was added into each culture dish. Cells were incubated on ice for 10 minutes and scraped using a cell scraper. The lysate was pooled and centrifuged at 15,000 x g for 15 minutes at 4 °C. Protein estimation was performed using the Qubit™ 4 Flourometer (ThermoFisher Scientific, USA) using the Qubit™ protein broad range (BR) assay kit (Cat. No: A50668, ThermoFisher Scientific, USA) according to the manufacturer's protocol.

2.2 Immunoblotting

Immunoblotting was conducted by loading 40 µg of cell lysate onto 10 % SDS-PAGE gels and then resolved. These were transferred onto nitrocellulose membranes and blocked overnight with 5% BSA in TBS. Primary antibodies were added and incubated overnight at 4 °C, followed by addition of HRP-conjugated secondary antibody incubation for 2 hours at RT. Chemiluminescent detection was done using BioRad XRS+ chemidoc apparatus following the addition of SuperSignal™ West Femto Maximum Sensitivity Substrate (Cat. No: 34094, ThermoFisher Scientific, USA). The blots were analyzed using ImageJ 1.53 software and graphs plotted using GraphPad Prism 5.0 Software using Vinculin as normalization control. Membranes were stripped for reprobing using Restore™ Western Blot Stripping Buffer (Cat. No: 21059, ThermoFisher Scientific, USA), reblocked, reprobed with antibody and developed. The antibodies used to probe proteins include Rab 5 (Cat. No: 3547), Rab 7 (Cat. No: 9367), Rab 9a (Cat. No: 5118), Rab 11 (Cat. No: 5589) and Vinculin (Cat. No: 13901), all from Cell Signaling Technologies, USA.

3. Results

3.1 Changes in morphology of Neuro2A cells upon okadaic acid (OA) treatment by light cell microscopy

The morphological changes induced by okadaic acid (100 nM) in Neuro2a cells with 4 hours of okadaic acid treatment was assessed, it was observed that the morphology of N2A cells changed with okadaic acid treatment and cells acquired round shape at concentration 100 nM (Fig: 1 D).

3.2 Effect of Okadaic Acid on Rab protein expression in Neuro2a cells by Immunoblotting

To determine how tau-related stress influences endosomal trafficking, Neuro2A cells were treated with okadaic acid (OA; 100 nM) for 4 hours, and the protein expressions of Rab5, Rab7, Rab9, and Rab11 were evaluated by Western blotting.

OA exposure led to a marked reduction in Rab5 expression**,** indicating impaired early endosome formation and maturation (Figure 2 A and A'). Similarly, Rab7 expression was also significantly decreased following OA treatment (Figure 2 B and B'), suggesting a disruption in the transition from early to late endosomes.

In contrast, proteins involved in recycling and late endosomal pathways showed the opposite trend. Rab9 expression was increased at the 4-hour time point (Figure 2 C and C'), pointing toward enhanced late endosome accumulation or retrograde trafficking to the Golgi. Additionally, Rab11 expression was increased after OA exposure (Figure 2 D and D'), reflecting a shift toward heightened recycling-endosome activity.

Overall, the Western blot results demonstrate that okadaic acid suppresses early and intermediate endosomal markers (Rab5 and Rab7) while promoting proteins associated with recycling and late endosomal trafficking (Rab9 and Rab11). This suggests that tau-mediated stress skews vesicular transport away from early endosome maturation and toward recycling and retrograde pathways.

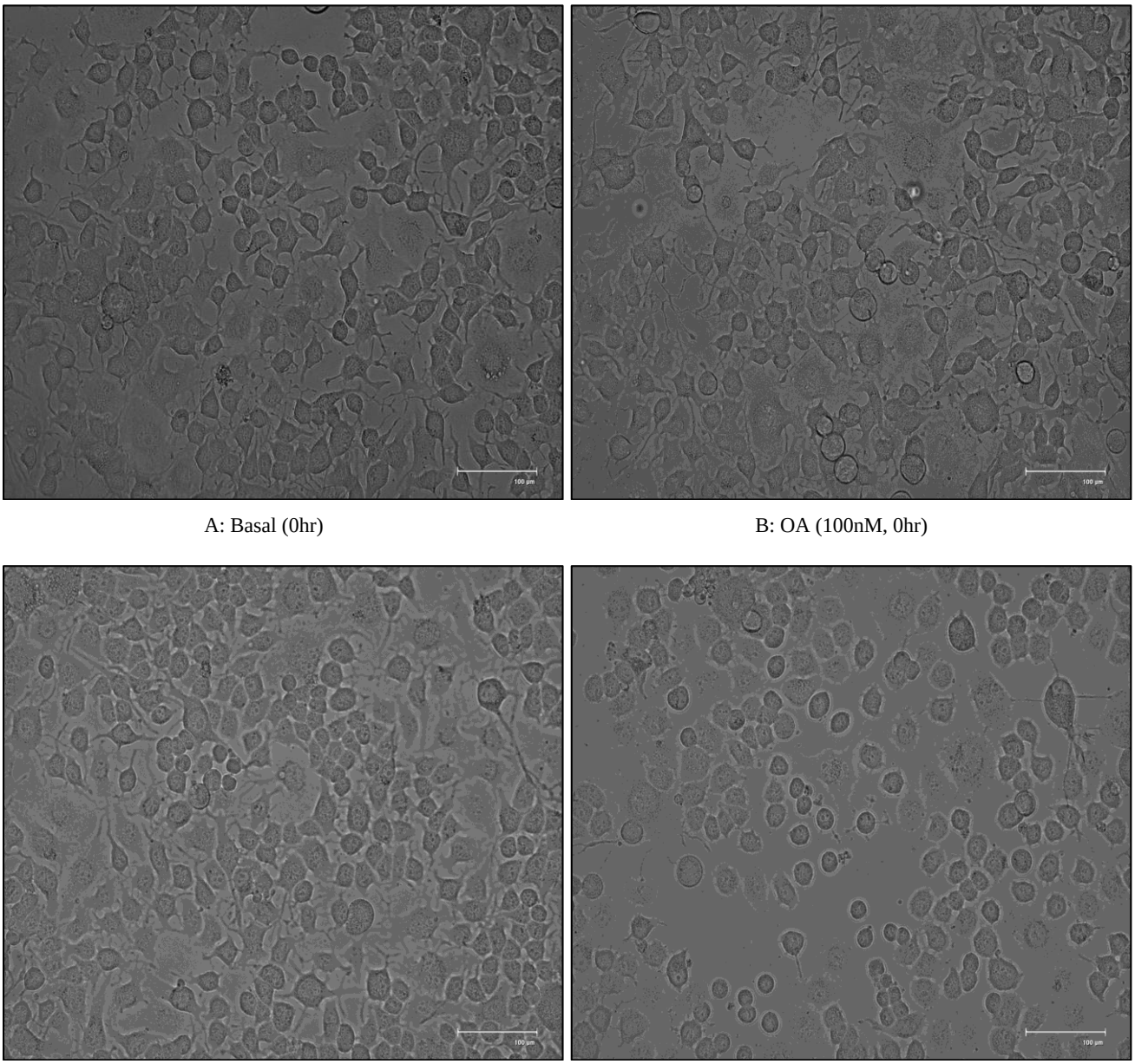
4. Discussion

Alzheimer's disease (AD) is marked by the accumulation of hyperphosphorylated tau, which destabilizes microtubules and disrupts intracellular transport [17]. Okadaic acid (OA), a well-established inhibitor of PP1 and PP2A, effectively induces tau hyperphosphorylation and mimics several pathological features of AD [16]. Using this model, we examined how tau-mediated stress alters the expression of Rab GTPases that regulate endocytosis and vesicular trafficking.

Because microtubules guide the movement of membrane-bound vesicles, tau-induced cytoskeletal instability is expected to impair endosomal maturation. Consistent with this, OA treatment resulted in reduced expression of Rab5 and Rab7—key regulators of early and late endosomal trafficking. Rab5 governs early endosome fusion and signalling, while Rab7 mediates the transition from early to late endosomes and supports autophagosome-lysosome fusion [9, 13]. Decreased levels of these proteins suggest impaired endosomal progression and compromised degradative capacity under tau-hyperphosphorylated conditions.

In contrast, Rab9 expression increased following OA exposure. Rab9 is essential for retrograde transport from late endosomes to the trans-Golgi network and contributes to maintaining late endosome structure [11]. Its upregulation may reflect compensatory remodelling of late endosomal dynamics in response to impaired early trafficking. Elevation of Rab11, a marker of recycling endosomes, further points toward enhanced recycling activity, possibly as an adaptive response to disrupted endosomal-lysosomal pathways.

Together, these changes indicate that tau hyperphosphorylation skews vesicular trafficking away from early and late endosomal maturation and toward recycling and retrograde transport. Such shifts may contribute to the vesicular dysfunction, impaired cargo degradation, and synaptic instability observed in AD [18]. Further studies are needed to clarify how tau-induced Rab dysregulation influences lysosomal biogenesis and neuronal homeostasis.



A: Basal (0hr) B: OA (100nM, 0hr)

C: Basal (4hr) D: OA (100nM, 4hr)

Fig 1: Representative images showing morphological changes induced by okadaic acid in Neuro 2A cells

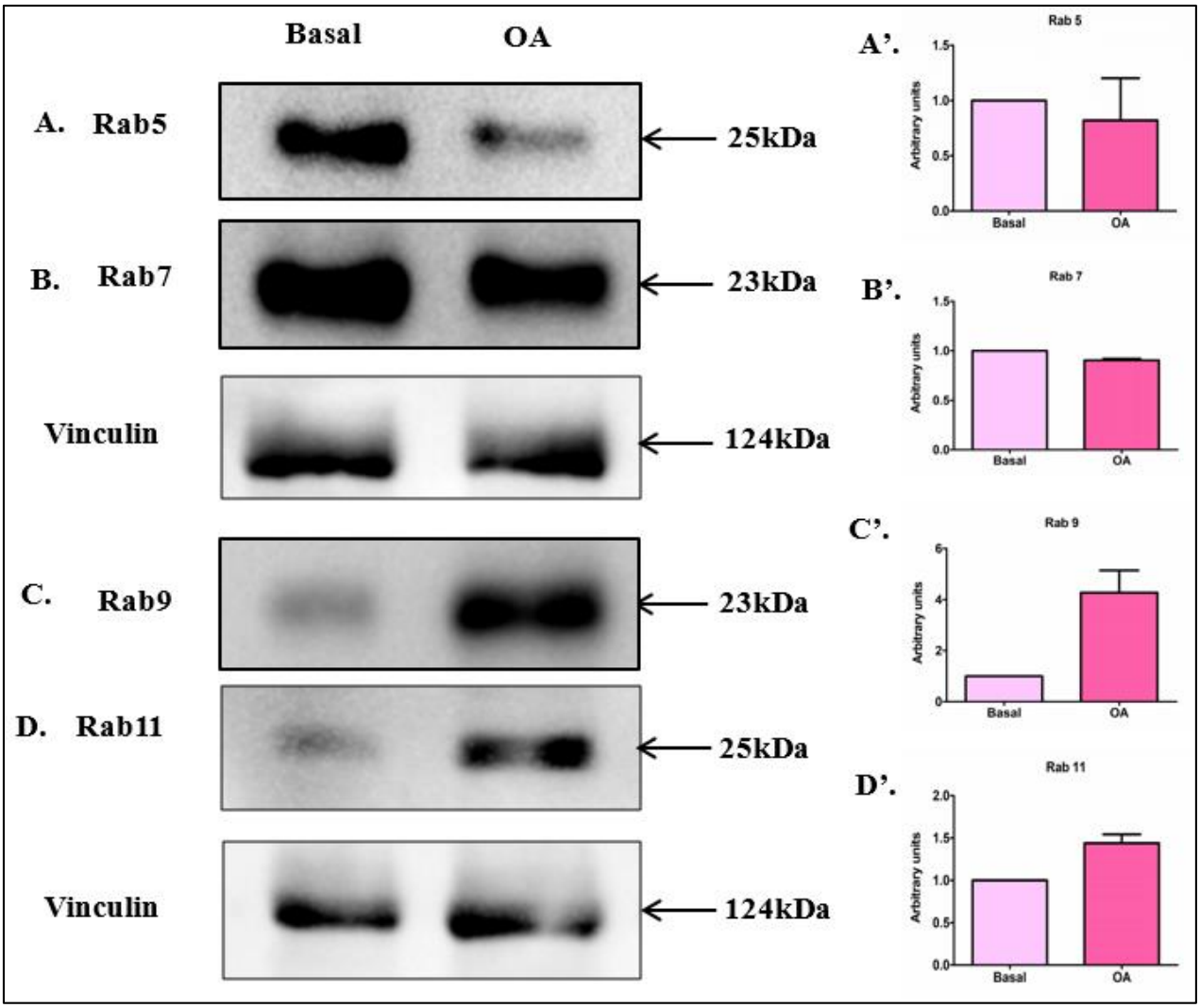


Fig 2: Representative immunoblots showing changes in Rab5, Rab7, Rab9 and Rab11 upon 4 hours of okadaic acid treatment in Neuro 2a cells. Vinculin was used as loading controls. The band intensities (target protein/Vinculin) were calculated using Image J (NIH) software.

5. Acknowledgments

ARN was supported by Council of Scientific and Industrial Research (CSIR) by the grant of Junior Research Fellowship (JRF). The study was supported by the grant of SERB-POWER, New Delhi, and Plan funds (2021-22, 2022-23), University of Kerala.

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