ISN Committee for Aid and Education in Neurochemistry (CAEN)
CATEGORY 1B: Research supplies for use in the applicant's home laboratory

Report by Dr. Akash Gautam

Project title: Analysis of Arc interacting proteins during amnesia and its restoration

Project start date: July 2017

Project end date: July 2018

Overview: Activity-regulated cytoskeletal-associated protein (Arc) is a member of immediate early gene family. Recent studies have shown its involvement in essentially every known form of synaptic plasticity (Bramham et al., 2010). Arc protein is highly expressed in dendrites, the postsynaptic density and the nucleus. In hippocampal neurons, Arc overexpression increases spine density *in vitro*, and disruption of Arc decreases spine density *in vivo* (Peebles et al., 2010). The exact role of Arc protein in various cognitive diseases is still elusive. For e.g. Palop et al., 2005 found that Arc expression decreases due to increased levels of Alzheimer's-related human amyloid precursor protein (hAPP) in transgenic mice expressing hAPP. Also, Arc forms complex with several protein products and few of those are linked to genes which are known to cause schizophrenia (Fromer et al., 2014 and Purcell et al., 2014). In our previous study too, we have shown the involvement of Arc in scopolamine-induced amnesia and its restoration with leaf-extract of *Withania somnífera* (i-Extract) in the hippocampus of mouse (Gautam et al., 2013; Gautam et al., 2016). The diminished level of Arc mRNA and protein in amnesic condition disrupts the morphology as well as density of mushroom shaped dendritic spines, which in turn is restored by treatment with i-Extract. But, the exact molecular mechanism by which Arc protein modulates this neuronal structure during amnesia and its restoration is unknown.

Hypothesis: According to our hypothesis, Arc mediates in the memory related disorder through the recruitment of a number of other crucial proteins. Therefore, analysis of Arc interacting proteins in mouse hippocampus during amnesia and its restoration is required.

Methods:
Animals and drug treatment

Male Swiss albino mice of 12±2 weeks were kept at 24±2^{0}C under 12 h light and dark schedule with *ad libitum* standard mice feed and drinking water. To analyze the interacting proteins of Arc during amnesia and its restoration, mice were divided into three groups: (i) Control- mice injected with normal saline (SA), (ii) Amnesic- mice injected with scopolamine (SC) and (iii) Memory restored- mice treated with i-Extract followed by scopolamine (i→SC). Saline (0.9%) or scopolamine hydrobromide (3mg/kgbw) dissolved in normal saline was injected intraperitoneally, whereas i-Extract (200 mg/kgbw) dissolved in 0.05% DMSO was fed orally 1 h prior to scopolamine injection. After seven days of daily treatment, the hippocampus was dissected out from each mouse after 3 h of final dose administration.

Preparation of protein lysate

A 10% homogenate of hippocampus was prepared in ice cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitors. The homogenate was centrifuged at 10,000xg for 15 min at 4^{0}C. The supernatant was collected and protein concentration was determined by Bradford method (1976).

Co-immunoprecipitation

The protein homogenate (100 µg) was incubated with 50 µl protein A Sepharose beads in final 500 µl immunoprecipitation buffer (0.02 M NaH_2PO_4, 0.15 M NaCl, pH 8.0) at 4^{0}C for 2 h and then centrifuged at 1000xg for 1 min at 4^{0}C. The pre-cleared supernatant was incubated overnight with 1 µg of anti-Arc in immunoprecipitation buffer. The next morning, 50 µl proteins A Sepharose bead was added to it and incubated again for 2 h at 4^{0}C with constant shaking. The unbound proteins were removed from the beads by washing thrice with wash buffer (40mM HEPES, pH 7.5; 100mM NaCl; 1 mM EDTA; 1 µl/mL protease inhibitor cocktail). Finally, the bound proteins were denatured by boiling with 2xSDS sample buffer. The interacting proteins were resolved by 10% SDS–PAGE, and detected with silver stain as mentioned in manufacturer's instructions. To analyze Co-IP bands, the signal intensity of each stained band was calculated as Integrated Density Value (IDV) by spot densitometry tool AlphaEaseFC software (Alpha Innotech Corp., USA).

MALDI-TOF-MS

The samples for MALDI-TOF-MS were prepared as described earlier by Kumar and Thakur (2014). The trypsin digested samples were analysed at the proteomics facility of the University of Hyderabad, Hyderabad. Finally, the obtained raw data was searched in MASCOT web server

(Matrix Science; http://www.matrixscience.com) by means of Biotools version 3.2 (Bruker Daltonics, Germany).

Western Blotting

Final confirmation of interacting proteins will be done by Western blotting using the primary and secondary antibodies. This work is still under the process.

Statistical analysis

Each experiment was repeated thrice (n = 3 × 3 = 9 mice/group). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by post hoc test of LSD method through PASW Statistics for Windows (version 18) for all parameters. Results were expressed as mean ± S.E.M. and p-values <0.05 were considered as statistically significant.

Results:

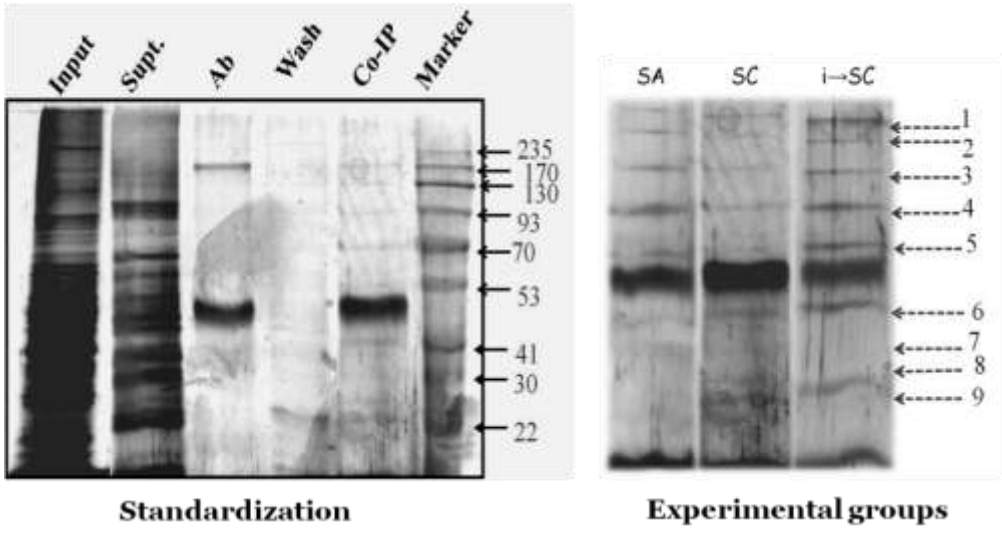
Co-IP followed by MALDI-MS/MS analysis identified nine Arc interacting proteins

With the optimized conditions for Arc, Co-IP gel showed nine different distinct protein bands (Fig. 1a). Predicted molecular weight of Arc interacting partners was in the range of 14–160 kDa. MALDI-MS/MS analysis identified these proteins as Grin2b, Cyfip1, Grin1, AMFR, Snx9, Krt15, Homer1, unnamed protein and Myl7. The summary of these proteins has been provided in Table 1.

Table 1- MALDI-MS/MS analysis of co-imunoprecipitated Arc interacting proteins

Band No.	Accession number and protein name	MW (kD)	Function(s)
1	Q01097 (*Grin2b*) Glutamate receptor ionotropic, NMDA 2B	160	NMDA receptor subtype of glutamate-gated ion channels with high calcium permeability and voltage-dependent sensitivity to magnesium.
2	Q7TMB8 (*Cyfip1*) Cytoplasmic FMR1-interacting protein 1	139	Component of the CYFIP1-EIF4E-FMR1 complex which binds to the mRNA cap and mediates translational repression. Plays a role in axon outgrowth. Binds to F-actin but not to RNA.
3	P35438 (*Grin1*) Glutamate receptor ionotropic, NMDA 1	111	This protein plays a key role in synaptic plasticity, synaptogenesis, excitotoxicity, memory acquisition and learning.

4	Q9R049 (*AMFR*) E3 ubiquitin-protein ligase autocrine motility factor receptor	84	E3 ubiquitin-protein ligase that mediates the polyubiquitination of a number of proteins such as CD3D, CYP3A4, CFTR and APOB for proteasomal degradation.
5	Q91VH2 (*Snx9*) Sorting nexin-9	63	Plays a role in endocytosis via clathrin-coated pits, but also clathrin-independent, actin-dependent fluid-phase endocytosis.
6	Q61414 (*Krt15*) Keratin, type I cytoskeletal 15	49	In the absence of KRT14, makes a bona fide, but ultra-structurally distinct keratin filament network with KRT5.
7	Q9Z2Y3 (*Homer1*) Homer protein homolog 1	39	Postsynaptic density scaffolding protein. It aids the coupling of surface receptors to intracellular calcium release.
8	Q3UF90 (unnamed) Putative uncharacterized	21	Not known
9	Q9QVP4 (*Myl7*) Myosin regulatory light chain 2	14	Motor protein, Muscle protein



(a) (b)

Fig. 1. Co-IP analysis of Arc interacting proteins in the hippocampus of mice during amnesia and its restoration. (A) 10% silver stained SDS-PAGE showing optimization of Co-IP for Arc

interacting proteins [Input protein (Input), Supernatant protein (Supt.), Arc antibody (Ab), final wash of Co-IP (wash), Co-immunoprecipitated protein (Co-IP) and Marker]. Anti-Arc protein of ~50 kD was excluded from further analysis. (B) Co-IP proteins in the hippocampus of control (SA), scopolamine-induced amnesic (SC) and pre-treated with i-Extract (i→SC) groups.

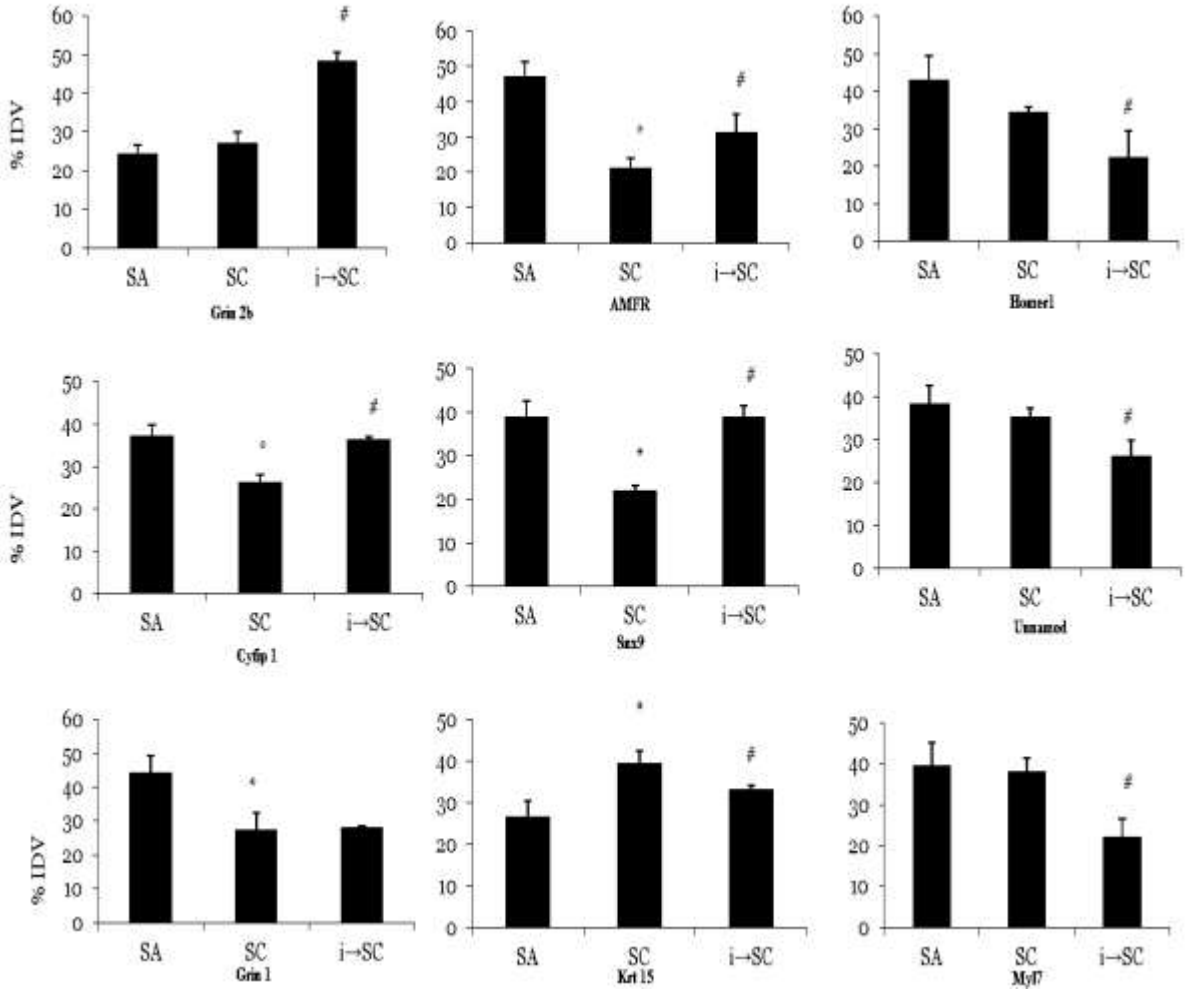


Fig. 2. Interaction level of each Co-IP protein plotted in terms of per cent relative densitometric analysis (% IDV) of each band resolved on the gel.

Table 2- Function and amino acid length of putative Arc interacting proteins, obtained by STRING at the confidence score > 0.5

Protein (amino acid length)	Function	Score
BDNF (257 aa)	Regulator of synaptic transmission and plasticity	0.892
SH3GL1 (368 aa)	Implicated in endocytosis	0.803
GRIN2B (1482 aa)	NMDAR subtype of glutamate-gated ion channels	0.764
CAMK2B (666 aa)	Function in long-term potentiation	0.673
CYFIP1 (1253 aa)	Involved in axon outgrowth. Binds to F-actin	0.669
GRIN1 (959 aa)	Plays a key role in synaptic plasticity	0.668
SELH (116 aa)	Involved in a redox-related process	0.625
PRMT6 (378 aa)	Mediates the asymmetric dimethylation of histone H3	0.556
ENSMUSG00000038005(346 aa)	RIKEN cDNA 2700029M09 gene	0.556
CCS (274 aa)	Delivers copper to copper zinc superoxide dismutase	0.556
CAMK2A (489 aa)	Function in long-term potentiation	0.553

Interaction level of Arc interacting proteins varies differentially during amnesia

Co-IP analysis showed that Arc interacting proteins varied differentially with respect to amount and degree of interaction in the hippocampus of mice during amnesia and its restoration (Fig. 1b). Out of these nine interacting partners, four proteins (Cyfip1, Grin1, AMFR, and Snx9) showed significant decrease and one protein (Krt15) showed significant increase in their level of interaction in scopolamine-induced amnesia as compared to control (Fig. 2). On the other hand, the remaining four proteins (Grin2b, Homer1, unnamed protein and Myl7) showed no significant change between control and amnesia. Pre-treatment with i-Extract attenuated the scopolamine induced changes in the interaction level of Cyfip1, AMFR, Snx9, and Krt15. Pre-treatment with i-Extract also increased the interaction level of Arc and Grin2b, but decreased the interaction level of Arc with Homer1, unnamed protein and Myl7 as compared to the scopolamine-induced

amnesic mouse group. The analysis through STRING showed 11 interacting partners of Arc protein, based on homology, neighbourhood, co-expression, gene fusion, co-occurrence, experiments, databases and text mining (Table 2). Out of these, we found three proteins i.e. Grin2b, Cyfip1, and Grin1 in our study too.

Conclusion:

We obtained different interacting partners of Arc as well as their modulation during amnesia and its restoration in the hippocampus of mouse. However, to validate or confirm the identity of these proteins, we are still continuing this study by performing Western Blotting using the specific antibodies.

Acknowledgements: I am highly thankful for the grants from the *ISN Committee for Aid and Education in Neurochemistry*.

REFERENCES

- Bramham, C.R., Worley, P.F., Moore, M.J., Guzowski, J.F., 2008. The immediate early gene Arc/Arg3.1 : regulation, mechanisms, and function. J. Neurosci. 28, 11760-11767.
- Fromer, M., Pocklington, A.J., Kavanagh, D.H., et al., 2014. De novo mutations in schizophrenia implicate synaptic networks. Nature, 506, 179-184.
- Gautam, A., Kaul, S.C., Thakur, M.K., 2016. Alcoholic Extract of Ashwagandha Leaves Protects Against Amnesia by Regulation of Arc Function. Mol. Neurobiol., 53, 1760-1769.
- Gautam, A., Wadhwa, R., Thakur, M.K., 2013. Involvement of hippocampal Arc in amnesia and its recovery by alcoholic extract of Ashwagandha leaves. Neurobiol. Learn. Mem., 106, 177-184.
- Gilbert, D.J., Jenkins, N.A., Lanahan, A.A., Worley, P.F., 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 14, 433-445.
- Kumar, A., Thakur, M.K., 2014. Analysis of Presenilin 1 and 2 interacting proteins in mice cerebral cortex during development. Int. J. Dev. Neurosci. 38, 138-146.
- Link, W., Konietzko, U., Kauselmann, G., Krug, M., Schwanke, B., Frey, U., Kuhl, D., 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc. Natl. Acad. Sci. U.S.A. 92, 5734-5738.
- Palop, J.J., Chin, J., Bien-Ly, N., Massaro, C., Yeung, B.Z., Yu, G.Q., Mucke, L., 2005. Vulnerability of dentate granule cells to disruption of arc expression in human amyloid precursor protein transgenic mice. J. Neurosci., 25, 9686-9693.
- Peebles, C.L., Yoo, J., Thwin, M.T., Palop, J.J., Noebels, J.L., Finkbeiner, S., 2010. Arc regulates spine morphology and maintains network stability in vivo. Proc. Natl. Acad. Sci. U.S.A. 107, 18173-18178.
- Purcell, S.M., Moran, J.L., Fromer, M., et al., 2014. A polygenic burden of rare disruptive mutations in schizophrenia. Nature, 506, 185-190.
- Rich, M.M., Wenner, P., 2007. Sensing and expressing homeostatic synaptic plasticity. Trends Neurosci. 30, 119-125.

Financial report:

S.No.	Item and Quantity purchased	Price (USD)
1	Antibodies: primary and secondary (10)	2500
2	PVDF membrane, Protein A/g beads, and general biochemicals & plastic-wares for co-IP and WB experiments	2000
3	MALDI-TOF-MS charges	500
	Total expenses	**5000**