Copine-6 immunoreactivity identifies a novel population of mature axonless neurons bordering the rostral migratory stream in rodent brain

Ruth Helen Faram[1], Kristina Detzner, Louise Kay[2], Peter Magill[1], Peter Somogyi[1], Jeff McIlhinney[1]

1, MRC Anatomical Neuropharmacology Unit, University of Oxford. 2, Department of Pharmacology, University of Oxford

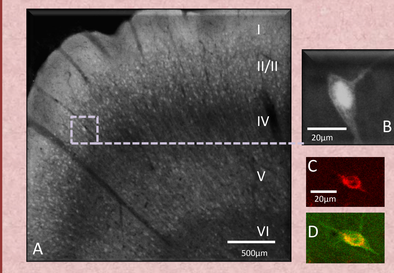
Introduction.

The Copines are a family of Ca^{++}-dependent phospholipid binding proteins (Creutz, et al 1998), with the ability to translocate to the plasma membrane in mammalian cells following an increase in intracellular calcium (Perestenko et al, 2010). The physiological function of the copines remains unclear. Copine-6 is a 'neuron specific' member, with mRNA expression increasing in the hippocampus following LTP and kindling (Nakayama et al, 1998, 1999). To investigate copine-6 protein expression, a rabbit polyclonal antibody was raised against copine-6 and its specificity established. This was then used in immunohistochemical studies.

Copine-6 is strongly expressed in specific neuronal populations of the rodent brain

Cortex :

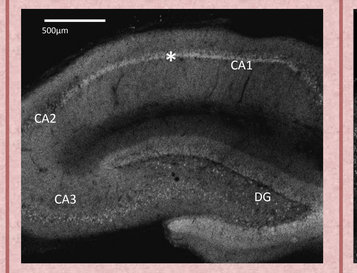
- Layer II/III pyramidal cells (some but not all, immunopositive along the somatic and dendritic plasma membrane, including dendritic spines of strongly immunopositive cells).
- Some layer II/III pro-CCK+ interneurons of the cortex



A: The regional expression of copine-6 immunoreactivity in the cortex. Note how the expression differs between layers, with strong pyramidal cell expression in layers II/III. **B:** Copine-6 immunoreactive interneuron within the layer II/III. **C:** Pro-CCK reactivity in copine-6 immunopositive interneuron in layer II/III. **D:** Merge, (copine-6 green, pro-CCK red). These cells are sparse, the majority are positive for pro-CCK.

Hippocampus:

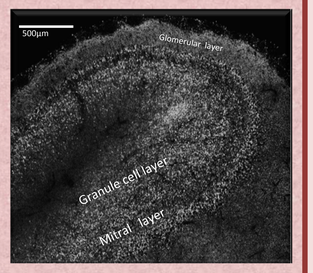
- Some pyramidal cells of CA1, 2 and 3
- Some granule cells of the dentate gyrus



The regional expression of copine-6 in the hippocampus. The copine-6 immunoreactivity is strongest in CA1 pyramidal cell layer (*), but note the laminar expression throughout, perhaps relating to input/output pathways of connecting neurons.

Olfactory Bulb:

- Cells within the granule and mitral cell layer

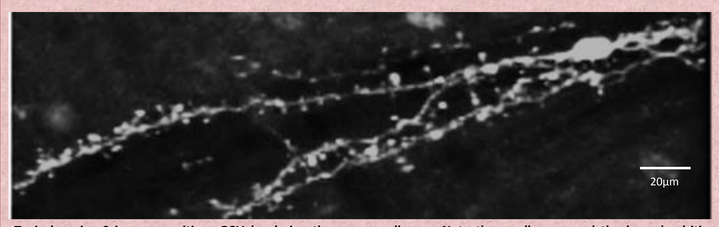


The regional expression of copine-6 in the main olfactory bulb. Most, but not all, cells in the granular and mitral cell layers are copine-6 immunopositive. Some of the copine-6 immunopositive granule cells are also calretinin postive, and lack an axon typical of the granule cell interneuron population.

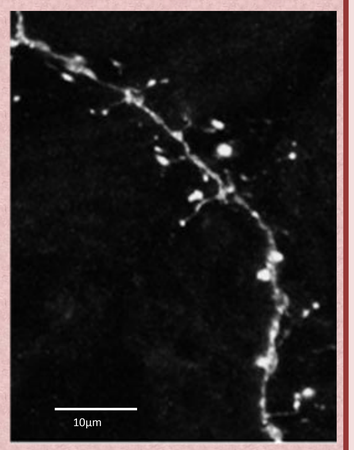
Rostral and caudal corpus callosum:

- An unusual population of small neurons aligned with rostral and caudal corpus callosum, bordering striatum and hippocampus
- The cells have a small soma (~10µm) with long, bipolar dendritic arborisations ornamented with multiple 'spine-like' protrusions
- Strongly and homogenously immunopositive throughout the cytoplasm
- Mature, **axonless** neuronal phenotype: similar to olfactory bulb granule cell so will be called **'Granule-cell-like Interneurons', GCLIs.**

***A similar cell phenotype has been identified by Monyer et al (2011)**



Typical copine-6 immunopositive GCLI bordering the corpus callosum. Note the small soma and the long dendritic arborisations



A copine-6 immunopositive dendrite with prolific spine-like protrusions

Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J., and Cohen, J. (1998). The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from Paramecium to humans. The Journal of Biological Chemistry 273, 1393-1402

Monyer, H., Le Magueresse, C., Alfonso, J., Khodosevich, K., Arroyo Martín, A., Bark C. (2011). "Small Axonless Neurons": Postnatally Generated Neocortical Interneurons with Delayed Functional Maturation. The Journal of Neuroscience, 31(46): 16731-16747.

Nakayama, T., Yaoi, T., Yasui, M., and Kuwajima, G. (1998). N-copine: a novel two C2-domain-containing protein with neuronal activity-regulated expression. FEBS letters 428, 80-4.

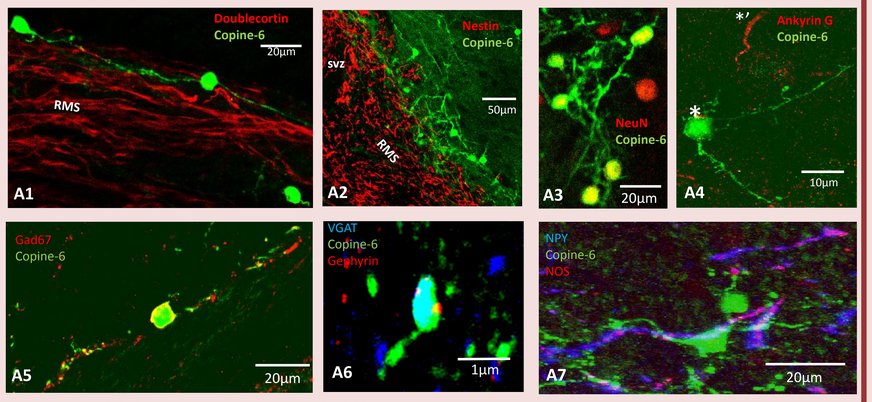
Nakayama, T., Yaoi, T., and Kuwajima, G. (1999). Localization and subcellular distribution of N-copine in mouse brain. Journal of neurochemistry 72, 373-9

Perestenko, P. V., Pooler, A. M., Noorbakhshnia, M., Gray, A., Bauccio, C., & Jeffrey McIlhinney, R. A. (2010). Copines-1, -2, -3, -6 and -7 show different calcium-dependent intracellular membrane translocation and targeting. The FEBS journal, 277(24), 5174-89.

Aims, Methods and Results

The copine-6 'granule cell like interneurons' (GCLIs) in the white matter were characterised by immunohistochemistry and light confocal microscopy

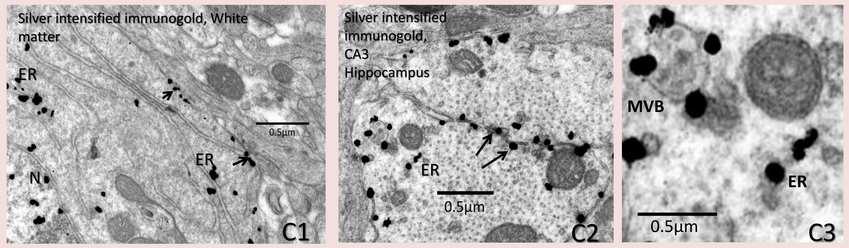
The copine-6 GCLIs border migrating immature neurons and newborn neuroblasts in the rostral migratory stream (RMS). The copine-6 GCLIs are mature GABAergic neurons that lack an axon. All copine-6 GCLIs are immunonegative for glial markers (see additional folder). They make putative synaptic contact by the dendritic protrusions, and can be in close proximity to other, peptidergic neurons, in the periphery of the white matter.



A1: Doublecortin+ cells (red) migrating in the rostral migratory stream (RMS), following neurogenesis at the subventricular zone. The copine-6 cells (green) are located close to, but on the periphery of the migrating cells. **A2**: Copine-6 cells are also peripheral to nestin + newborn neuroblasts (red) at the level of the subventricular zone (SVZ) and RMS. **A3**: All copine-6 cells in the white matter are coimmunoreactive for NeuN+ (red), indicating a mature neuronal phenotype. **A4**: Copine-6 cell (*) in the white matter lacking immunoreactivity for Ankyrin-G (red), a marker of the axon initial segment (AIS). (*'): a positive AIS from a copine-6 immunonegative cell. See additional folder for more images. **A5**: All copine-6 cells in the white matter are immunopostive for GAD67, indicating a GABAergic phenotype. **A6**: Putative synaptic contact on a copine-6 immunopostive dendritic protrusion (VGAT+ (blue, presynaptic) and Gephyrin+ (red, postsynaptic) onto an unknown structure. **A7**:Neuropeptide-Y(NPY, blue) / NOS (red) expressing cells which are also in the periphery of the white matter are located in close proximity to the copine-6 cells.

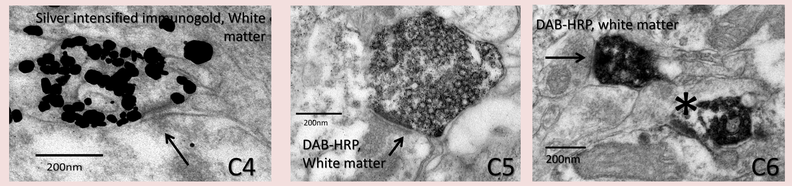
The distribution of copine-6 protein within GCLIs of white matter, and hippocampal pyramidal cells was examined using DAB-HRP and silver intensified nanogold particles and electron microscopy.

Copine-6 was localised at the plasma membrane, and the membrane of the endoplasmic reticulum (ER) and multivesicular bodies (MVB) in all immunoreactive cells (GCLIs and CA3 pyramidal cells).



C1. Immunogold/silver particles are located at the edge of the plasma membrane (arrows) in copine-6 immunoreactive GCLI on the periphery of the corpus callosum. Nuclear reactivity was diffuse and an artefact cannot be excluded, and will be tested in control reactions. **C2**. Plasma membrane (arrows) and ER associated immunogold/silver particles in CA3 pyramidal cell dendrites in the hippocampus. This demonstrates the same copine-6 protein distribution in two different cell populations.**C3**: Copine-6 clusters around the membrane of a multivesicular body. This could be seen in Copine-6 immunopositive GCLIs and CA3 pyramidal cells.

In the white matter, all labelled varicosities of GCLIs made close apposition with other dendrites, boutons and/or glia. All appositions were either non-synaptic or postsynaptic and were demonstrated by immunogold particle labelling and DAB-HRP. So far, no evidence has been found of copine-6-positive spine or varicosity forming a presynaptic junction.



C4. Silver intensified gold immunolabelling of a copine-6+ dendrite in the white matter; it forms a junction with an unidentified process(arrow). No clear accumulations of vesicles can be identified, thus it is likely to be an adherens junction. **C5**. A postsynaptic copine-6 immunoreactive spine head, laden with synaptic vesicles, **receives** a synaptic junction from a bouton of unknown origin.**C6**. Two copine-6+ dendritic varicosities (DAB-HRP); the one on the left is postsynaptic (arrow) to a presynaptic terminal. The one on the right, has an invaginated protrusion from another cell (asterisk*), which was followed in serial sections.

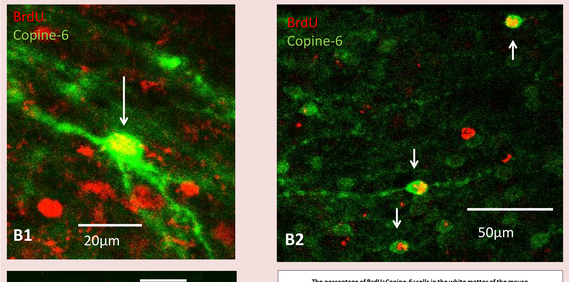
BrdU birthdating was used for developmental analysis of the copine-6 GCLIs

Because the Copine-6 GCLIs border a region of adult neurogenesis (SVZ, RMS) it was important to investigate when the cells were born. BrdU birthdating during embryogenesis and adult neurogenesis was conducted in both mice and rats.

The Copine-6 GCLIs are born during embryogenesis, with a peak in cell production during embryonic days 13 and 14. The Copine-6 GCLIs are **not** born in the adult brain.

The copine-6 GCLIs remain in the same location in the white matter throughout development and continue to be in this location in the adult brain. The embryonic data shows that the GCLIs remain in the white matter following birth, which supports previous data that they are not migrating (doublecortin immunonegative) and are indeed mature.

Methods: For embryogenic birthdating, 200mg/kg BrdU was injected intraperitoneally into pregnant mice, at embryonic stages e10, e12, e13, e14 and e15 (1 pulse per mouse). Pups were delivered naturally and were sacrificed at various developmental timepoints. For adult birthdating, 200mg/kg BrdU was injected into male sprague-dawley adult rats (250g+) (1 pulse per rat) and rats were sacrificed at different days following dosing. HCl antigen retrieval was used for immunoreaction of BrdU.



B1: A copine-6 GCLI (arrow) bordering the white matter of a postnatal day 5 mouse, following BrdU injection at embryonic day 14. The cell is immunoreactive for BrdU. **B2**: BrdU injection at embryonic day 13, sacrificed at postnatal day 26, 3 labelled GCLIs bordering the white matter (arrows) **B3**: BrdU injection into the adult rat, sacrificed 24 hours later. Copine-6 GCLIs were immunonegative for BrdU. The same result was found following 12 and 28 days incubation with BrdU **B4**: GCLIs in the white matter were counted and those BrdU+ were calculated as a percentage of the total number. Cells were counted from at least 12 sections per animal. All postnatal sacrifice timepoints yielded the same trend. Copine-6+ GCLIs are present at all developmental stages and appear to be born at the same embryonic timepoints, therefore suggesting that they remain in the white matter once born.

Conclusions

- The subcellular localisation of copine-6 on the plasma membrane, and on the membrane of the ER and multivesicular bodies within the cytoplasm, suggests that the copine-6 protein may have a role in protein translocation, trafficking or turnover within the cell.
- The copine-6 GCLIs in the white matter are postsynaptic. The lack of evidence for presynaptic terminals, despite the presence of vesicles and their VGAT immunoreactivity, may suggest an alternative, non-synaptic, output signalling mechanism. Also, it is possible that the efferent synaptic junctions are small and unusual. The cells are in close proximity to NPY/NOS expressing neurons therefore they may be influenced by peptidergic mechanisms.
- The copine-6 GCLI cells originate during embryogenesis. The cells remain in the periphery of the corpus callosum, surrounding the RMS throughout development and to adulthood. The cells may have a specific role, perhaps guiding the migrating cells – but their presence in caudal corpus callosum suggests additional roles.