Supplemental Methods

Recurrent Hypoglycemia is Associated with Loss of Activation in Rat Brain Cingulate Cortex

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Immunohistochemistry (IHC)

Following transcardial perfusion with saline then fixative, brains were removed, post fixed in 10% formalin, pH 7.0, for 4h and then submerged overnight in 30% sucrose in DEPC-treated phosphate-buffered saline (DEPC-PBS). Brains were cut on a freezing microtome at 25□m (1:6) and stored in an antifreeze solution containing 30% ethylene glycol and 20% glycerol in DEPC-PBS at -20^{o}C.

Chromagenic and fluorescent IHC was modified from previous studies (1-4). Each step was preceded by PBS rinses. The sections were pre-treated with 0.3% hydrogen peroxide (Sigma) diluted in PBS for 30 minutes followed by a blocking step with 3% normal donkey serum (Equitech-Bio, Kerrville, TX) diluted in PBS with 0.25% Triton X-100 (PBT) for 1 hour. The tissue was incubated in rabbit primary antiserum for Fos (Calbiochem, La Jolla, CA; 1:10,000) in 0.3% normal donkey serum and PBT-azide (0.02% sodium azide in PBT) overnight at room temperature with gentle agitation. Next, the sections were incubated in biotinylated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA; 1:1,000) in 0.3% fresh donkey serum and PBT for 1 hour at room temperature, and then in avidin-biotin complex (ABC; Vector Elite kit; Vector laboratories, Burlinghame, CA; 1:500 in PBS) for 1 hour at room temperature. After rinsing, the sections were incubated in 0.04% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% hydrogen peroxide in PBS. The reaction was terminated after 5-7 minutes with successive rinses in PBS. The sections were mounted onto polysine slides, air-dried overnight, dehydrated in ascending concetrations of ethanol, cleared in xylenes and cover-slipped with mounting medium (Micromount, Surgipath, Peterborough, UK).

Adjacent sections of brain tissue were then processed determination of endogenous proteins for Neuronal Nuclei (NeuN) and Glial Fibrillary Acidic Protein (GFAP). Following the FOS-IR single label protocol above, tissue was extensively washed in PBS and then mouse anti-Neuronal Nuclei (NeuN; 1:1000,Chemicon) and rabbit anti-Glial Fibrillary Acidic Protein (GFAP; 1:1000, Chemicon) primary antisera were added and tissue was incubated for a further 12-16 hrs. After a PBS rinse, sections were transferred to a solution containing secondary antibodies Alexa Fluor-488 donkey anti-mouse (Invitrogen, 1:1000), and Alexa Fluor-568 donkey anti-rabbit (1:1000, Invitrogen) for 1 hour. The sections were mounted on gelatin coated slides, air-dried, and coverslipped with fluorescence Vectashield mounting medium (Vector). Immunofluorescence was observed under an epiofluorescence microscope Zeiss Axioskop2 with appreciate filter sets for Alexa Flour-488(excitation, 450-490 nm; emission, 514-625 nm) and Alexa Flour-568 (excitation, 530-585 nm; emission >615 nm).

Sections were examined using a Zeiss Axioskop II with attached CCD camera. Bregma levels were assigned with reference to the Rat Brain in Stereotaxic co-ordinates (5) and digital images captured using Axiovision 4.3 software (Carl Zeiss Vision). Images were merged (where appropriate) and optimised using Adobe Photoshop CS3 (Adobe systems Incorporated). Co-expression in dual-labelled cells was determined and counted manually.

Based on initial screening of the pattern of distribution of FOS-IR cells in the brain, we identified four cortical areas of interest for a detailed comparison: cingulate cortex 1 (Cg1; levels from bregma 1.80, 0.96, 0.36, -0.24, -0.84); cingulate cortex 2 (Cg2; levels from bregma 1.80, 0.96, 0.36, -0.24, -0.84); perirhinal cortex (PRh; levels from bregma -3.24, −3.84, −4.44, −5.04, -5.64); ectorhinal cortex (Ect; levels from bregma -3.24, −3.84, −4.44, −5.04, -5.64). We also examined the primary somatosensory cortex (S1; levels from bregma 1.80, 0.96, 0.36, -0.24, -0.84) as a control cortical area adjacent to the cingulate cortex. All bregma levels were assessed using the rat brain atlas of Paxinos and Watson (5^{th} edition). Immuno-positive cells for *c-fos* were counted across the entirety of the brain slice and counts for the different bregma levels were summated for each animal, which

were then averaged across all the animals in each respective group. This gave a representative 'total' c-fos count for each treatment which was used to produce figures and for statistical comparison.

AH treated brains processed for triple IHC for FOS-IR, the neuronal marker NeuN and the glial marker GFAP were assessed qualitatively given the majority of FOS-IR positive cells were co-expressed with NeuN, not GFAP.

References

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