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E-cadherin is required at GABAergic synapses in cultured cortical neurons

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ABSTRACT

Classical cadherins are cell adhesion molecules that are thought to contribute to the control of synapse formation, synaptic transmission, and synaptic plasticity. This is largely based on studies investigating the functions of N-cadherin at glutamatergic synapses, whereas other classical cadherins have hardly been examined at central synapses. We have now used a conditional knockout approach in cultured cortical neurons to address the role of E-cadherin mainly at inhibitory, GABAergic synapses. Cortical neurons were cultured from mouse fetuses carrying floxed E-cadherin alleles in homozygous configuration. E-cadherin knockout was induced in individual neurons by expression of an EGFP-Cre fusion protein. Immunocytochemical stainings for the vesicular GABA (VGAT) and glutamate (VGLUT1) transporters revealed a reduced density of dendritic GABAergic synapses in E-cadherin knockout neurons, whereas glutamatergic synapses were unaffected. Electrophysiological recordings of miniature and action potential-evoked, $GABA_A$ receptor-mediated postsynaptic currents confirmed an impairment of GABAergic synapses at the functional level. In summary, our immunocytochemical and electrophysiological analysis of E-cadherin knockout neurons suggested that E-cadherin signaling importantly contributes to the regulation of GABAergic synapses in cortical neurons.

Synaptic cell adhesion molecules play crucial roles during the formation and functional maturation of central synapses [2,3,6,28]. In addition, they have been proposed to regulate the synaptic vesicle cycle at mature synapses [6,11], and to control changes in postsynaptic spines during synaptic plasticity [5,18,21]. Both anterograde and retrograde signaling have been described to underlie the synaptic functions of transsynaptically interacting adhesion proteins [25,26,28].

Classical cadherins, such as N- and E-cadherin, are transmembrane adhesion proteins that homophilically interact across the synaptic cleft via extracellular cadherin domains. At their cytoplasmic part cadherins bind several types of catenins thus forming cadherin/catenin adhesion complexes [13,15,24,29]. N-cadherin is well known to be expressed at developing and mature glutamatergic synapses [2,8,30,33]. Functionally, N-cadherin is involved in the accumulation of synaptic vesicles during synapse formation [1,27,32], and in the transsynaptic regulation of the vesicle cycle at mature synapses [14,27]. Moreover, N-cadherin has been shown to be important for the formation and activity-dependent plasticity of dendritic spines [5,7,18,21,32].

In contrast to N-cadherin, a potential role of E-cadherin at central synapses has hardly been investigated, although the expression of E-cadherin at central hippocampal synapses has been demonstrated [9,31]. Moreover, cadherins other than N-cadherin have been proposed to be expressed at hippocampal GABAergic synapses [2,22]. To study the function of E-cadherin at central synapses, we used a knockout approach in cortical neurons. Because E-cadherin knockout mice are embryonically lethal [16], we performed a conditional E-cadherin knockout in cultured neurons derived from floxed E-cadherin mice [4]. The E-cadherin knockout was induced in individual neurons by low-efficiency transfection of an EGFP-Cre fusion protein expression vector. Subsequent immunocytochemical and electrophysiological analysis of individual E-cadherin knockout neurons revealed an important role of E-cadherin in the formation and function of a subpopulation of GABAergic synapses.

E17 mouse fetuses carrying floxed E-cadherin alleles in homozygous configuration were used to prepare standard cultures of cortical neurons. The creation of a homozygous mouse line carrying floxed E-cadherin alleles by gene targeting has been described previously [4]. Homozygosity of fetuses for floxed E-cadherin alleles was confirmed by PCR as described (primers pE10.2 und pE11as.2 [4]). Dissociated cortical neurons were cultured without a glial feeder layer on poly-L-ornithine coated coverslips/culture dishes as described previously [20,27]. E-cadherin expression in these cultures was confirmed at 7 days in vitro (DIV) by Western blotting (polyclonal rabbit antibody, Abcam; data not shown).

To induce the conditional knockout of E-cadherin in cultured cortical neurons, individual neurons were transfected

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with an EGFP-cre expression vector (pBS598 EF1alpha-EGFPcre, http://www.addgene.org; [10,17] at a low transfection efficiency using the Lipofectamine technique. Transfection of cortical cultures using Lipofectamine 2000 (Invitrogen) was performed at 4 DIV according to the manufacturer's instructions. For EGFP control transfection and for EGFP co-transfection experiments (vector ratio 1:5) involving immunocytochemistry the pEGFP-N1 expression vector (Clontech) was used. To check the co-transfection procedure, 2–3 days after transfection the expression of the Cre recombinase was analysed by fluorescence imaging of the EGFP-Cre fusion protein (Fig. 1). The EGFP-Cre fusion protein was mainly present in the nucleus and the cell soma, whereas the dendrites showed very little EGFP fluorescence. In these initial experiments co-transfection with a DsRed2 expression vector (pDsRed2-N1, Clontech, vector ratio 1:5) was used to visualize the soma and the dendrites of the transfected neurons. 3 days after co-transfection almost all (>97%) DsRed2 expressing neurons exhibited also EGFP-Cre expression in the nucleus (Fig. 1C).

To quantify the number of presynaptic vesicle clusters on transfected neurons, transfected (4 DIV) cortical cultures were immunostained at 7 DIV and 14 DIV with a primary antibody against VGAT to stain selectively GABAergic synapses, and against VGLUT1 to stain selectively glutamatergic synapses (polyclonal rabbit antibodies, Synaptic Systems). Immunocytochemistry was performed according to standard protocols as described previously [27] using a secondary, Cy3 conjugated antibody (goat anti-rabbit antibody, Chemicon). Fluorescence imaging of transfected neurons (EGFP fluorescence) and immunostained vesicle clusters (Cy3 fluorescence) was performed as described [27]. Using ImageJ software, maximum projection images were calculated from z-stacks. Images of immunostained vesicle clusters were then background subtracted, thresholded, and overlayed on EGFP images of transfected neurons to count the number of vesicle clusters on transfected cells (Fig. 2A–D). Means ± SEM were calculated and statistical analysis was done using Student's *t*-test.

Whole-cell patch-clamp recordings from transfected (at 4 DIV) cortical neurons were obtained as described previously [14]. Using live fluorescence microscopy, transfected neurons were identified by EGFP-Cre and EGFP expression (control cells), respectively. The intracellular solution contained 110 mM KCl, 0.25 mM $CaCl_2$, 10 mM EGTA, 20 mM HEPES, pH = 7.3. Holding potential was −60 mV. The extracellular solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH = 7.3. $GABA_A$ receptor mediated miniature PSCs ($GABA_A$ mPSCs) were recorded at 7 DIV and at 14 DIV in the presence of 1 μM TTX and 20 μM DNQX, and were completely blocked by addition of 7.5 μM gabazine. $GABA_A$ mPSCs were quantitatively analysed using the Mini Analysis software (Synaptosoft). $GABA_A$ PSCs evoked by presynaptic action potentials were recorded at 14 DIV in the presence of 20 μM DNQX, and were completely blocked by addition of 7.5 μM gabazine. Presynaptic action potentials were elicited by extracellular paired-pulse stimulation (100 ms interstimulus interval). For both E-cadherin knockout neurons and control neurons a stimulation strength of up to 30 V was applied to the patch pipette used for extracellular stimulation resulting in a maximal postsynaptic response. QX314 was added to the intracellular solution to block the generation of postsynaptic action potentials. Evoked $GABA_A$ PSCs were quantitatively analysed using pCLAMP software (Molecular Devices). Means ± SEM were calculated and statistical analysis was done using Student's *t*-test.

To investigate the role of the cell adhesion molecule E-cadherin in synapse formation and maintenance, we studied, whether a conditional knockout of E-cadherin affects the number of synapses in cultured cortical neurons. To induce and identify E-cadherin knockout neurons, we co-transfected the EGFP-cre expression vector together with an EGFP expression vector at 4 DIV to enable the visualization of the soma and the dendrites of the cre transfected neurons by fluorescence imaging of EGFP. Control cultures were transfected with the EGFP expression vector only. To determine changes in the number of GABAergic synapses, transfected cultures were immunostained for the vesicular GABA transporter

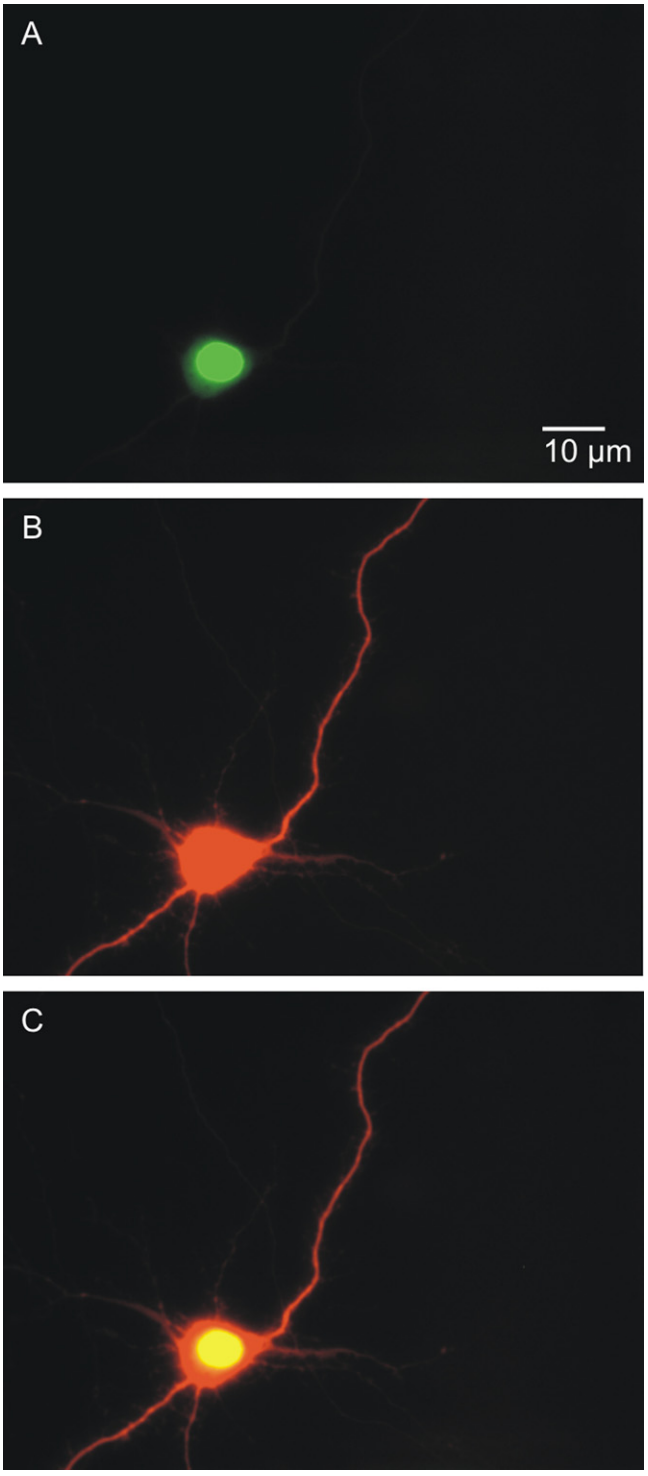

Fig. 1. Induction of E-cadherin knockout by expression of EGFP-Cre in cultured cortical neurons from homozygous floxed E-cadherin mice. Lipofectamine co-transfection of individual neurons with EGFP-cre and DsRed2 at 8 DIV. (A) EGFP-Cre fluorescence image two days after co-transfection. Note that the expression of EGFP-Cre is strongest in the nucleus. (B) DsRed2 fluorescence image two days after co-transfection to visualize the transfected neuron. (C) Overlay of EGFP-Cre fluorescence and DsRed2 fluorescence images. Scale bar, 10 μm.