A profile of the insulin receptor in cultured hippocampal slices

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Abstract

OBJECTIVES: Insulin receptors (IRs) are distributed in a region-specific fashion throughout the brain, and may play a role in processes related to learning and memory. The hippocampus, which participates in spatial memory formation, is one region in which the IR is abundantly expressed. Organotypic hippocampal slice cultures (OHSCs) are an in vitro model that permits the easy manipulation of growth conditions, yet retains much of the source structure's cytoarchitecture. To assess OHSCs as a model for the study of hippocampal IRs, ligand-binding and the expression and cellular distribution of the β-subunit (which transduces the insulin signal) were examined over time in culture.

DESIGN & RESULTS: Fluorescently conjugated insulin was used to assess neural insulin receptor binding, and revealed that labelling remained similar over three weeks in culture (a typical length of OHSC maintenance). Cross-linking of surface proteins helped to show that approximately half of β-subunits were found at the cell surface, and that this relative proportion remained stable over several weeks. In contrast, expression of the β-subunit protein progressively declined to a plateau approximately 60% less than that seen when the cultures were prepared.

CONCLUSIONS: Our results provide a foundation for subsequent studies to employ OHSCs to explore neural IRs; for instance, the dissonance between the progressive decline in expression of the IR β-subunit and the relative stability of receptor-mediated binding suggests the presence of an active process to hold steady the ability of cells to respond to insulin stimulation.

Abbreviations:

ACSF	- artificial cerebrospinal fluid
BS3	- bis(sulfosuccinimidyl) suberate
DIV	- days *in vitro*
INS-FITC	- insulin labelled with fluorescein isothiocyanate
IR	- insulin receptor
OHSC	- organotypic hippocampal slice culture
uINS	- unlabelled insulin

ORIGINAL ARTICLE

INTRODUCTION

The passage of peripheral insulin into the cerebrospinal fluid was initially reported more than forty years ago (Margolis & Altszuler 1967), and subsequent studies confirmed that insulin is indeed present within the brain (Havrankova *et al.* 1978a; Mielke & Wang 2011). A receptor for insulin has also been identified in neurones, and bears considerable structural similarity to its peripheral counterpart (Havrankova *et al.* 1978b; Mielke & Wang 2011). Whilst insulin acts primarily to facilitate glucose uptake within peripheral tissues, the use of glucose in many brain regions is thought to be largely mediated by an insulin-insensitive transporter (Simpson *et al.* 2008; although, see also Grillo *et al.* 2009). In addition, insulin is unevenly distributed within the brain (Banks 2004), and its receptor is expressed in a heterogeneous fashion (Schulingkamp *et al.* 2000). Taken together, these data provoke considerable interest regarding the role of neural insulin signalling.

The hippocampus is a relatively small structure embedded within the medial portion of the temporal lobe, and has generated considerable and sustained interest due to a role in the formation of event-related memories (Scoville & Miller 1957; Bird & Burgess 2008). The successful completion of a hippocampal-dependent memory task has been shown to modify expression of the hippocampal insulin receptor within rodents (Zhao *et al.* 1999; Dou *et al.* 2005), and the direct infusion of insulin into the hippocampus has been found to enhance performance within the same task (Moosavi *et al.* 2006), In addition, blockade of endogenous hippocampal insulin signalling with an antibody-like peptide was shown to impair memory performance (McNay 2007), whilst disruption of the IR through diet-induced insulin resistance altered cellular features of hippocampal function related to memory formation (Mielke *et al.* 2005b; Stranahan *et al.* 2008). As well, insulin has been shown to affect the cell-surface expression of several ligand-gated ion channels known to influence synaptic plasticity (a cellular correlate of learning and memory), including NMDA (Skeberdis *et al.* 2001), AMPA (Ahmadian *et al.* 2004), and $GABA_A$ (Wan *et al.* 1997) receptors. Collectively, the summarised evidence strongly suggests that signal transduction at the level of the neural IR contributes to the processes that allow for hippocampal-dependent memory.

Given the presumed importance of the IR to hippocampal function, we sought to examine essential characteristics of the receptor within a model system of the structure more amenable to subsequent cellular and molecular level analyses and interventions. Organotypic hippocampal slice cultures (OHSCs) are an *in vitro* model wherein tissue sections derived from early post-natal rodents are maintained upon a porous membrane at an interface between atmosphere and medium (Mielke *et al.* 2005a). Similar to dissociated neurones, interface OHSCs allow for growth and development to be easily monitored, yet, unlike cultured cells, they maintain much of the cytoarchitecture typical of the hippocampus. To profile the IR within OHSCs, as a foundation for subsequent studies, we assessed changes in three key measures over a typical three week culture period: ligand binding, the level of expressed β-subunit protein, and the surface density of the β-subunit.

MATERIALS AND METHODS

Organotypic hippocampal slice culture preparation

Organotypic hippocampal slice cultures (Mielke *et al.* 2005a) were prepared using procedures approved by the institutional animal care committee. For each culture, 6–8 Sprague-Dawley rat pups at post-natal day 8 were anaesthetised with ketamine (1 mg/pup; Bioniche, Belleville, ON, Canada), and placed over ice for 2 min. Following decapitation, brains were rapidly removed, and hippocampi dissected over ice. A McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, U.K.) was used to prepare 400 μm transverse slices, which were then placed in a dissecting solution consisting of Gey's balanced salt solution (Sigma, Oakville, ON, Canada; all subsequent reagents were from Sigma unless otherwise noted) supplemented with 35 mM glucose. Slices were examined under a dissecting microscope, and those considered suitable (generally 10–12 per animal) allowed to recover for 60–90 min at 4 °C. Each slice was then transferred to a sterile, humidified, semi-porous Millicell-CM insert (3–4 slices/insert; Millipore, Bedford, MA, USA) placed in a 6 well culture tray (Falcon, VWR, Mont-Royal, PQ, Canada) containing 1 mL medium/well. Medium consisted of 50% MEM with HEPES modification, 25% Hank's BSS, 25% heat inactivated horse serum, 1 mM glutamine, and 35 mM glucose. Slice cultures were maintained in a 5% CO_2 (balance air) humidified incubator at 36 °C, and complete media changes were done 1 day after plating and 3x/week thereafter.

To examine the developmental expression of proteins, 7 slices were pooled from each culture on the day of preparation, and 24 slices from the same culture were pooled after 7, 14, 21, and 28 days *in vitro* (DIV). Cultured slices were washed twice with cooled PBS, removed from membranes, and transferred to 400 μL of non-ionising lysis buffer [10 mM Tris, 25 mM EDTA, 100 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) NP-40, pH 7.40], supplemented with a protease inhibitor cocktail. Following manual homogenisation over ice, lysates were centrifuged at 1 000 × g for 10 min (4 °C), and supernatant protein concentration determined with a BioRad DC protein assay kit.

Fluorescence-based insulin binding assay

Cultured slices were placed over ice, and then washed twice with cooled (1–4 °C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124.0 NaCl, 3.0 KCl, 1.2 NaH_2PO_4, 1.0 $MgSO_4$, 2.0 $CaCl_2$, 26.0 $NaHCO_3$,

10.0 glucose, 10.0 HEPES, aerated with carbogen (95% O_2/5% CO_2), pH 7.37–7.43, 300–310 mOsm. After this, OHSCs were incubated for 15 min (in the dark with agitation at 4 °C) with either vehicle (ACSF) or insulin fluorescently labelled with fluorescein isothiocyanate (INS-FITC). Slices were then washed 4× with ACSF, removed from inserts, and added to 250 µL RIPA buffer [50 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) Na-DOC] supplemented with a protease inhibitor cocktail. Following manual homogenisation, 100 µL aliquots were added to a 96 well plate (Millipore) and fluorescence read via spectrophotometry (Cytofluor 2350 fluorescence platereader, Millipore; excitation: 485 nm, emission: 530 nm). In each experiment, two inserts with 3–4 slices/insert were used for every condition, and the mean fluorescence of vehicle-treated slices was subtracted from the mean value collected for slices treated with INS-FITC. In certain experiments, unlabelled insulin (uINS) was added to OHSCs during incubation with INS-FITC.

Cross-linking of surface proteins

OHSCs were placed over ice and then washed twice with cooled ACSF. Immediately prior to each experiment, the cross-linking solution was prepared by adding bis(sulfosuccinimidyl) suberate (BS^3; Pierce Biotechnology) to ACSF (1 mg/mL). Each insert was incubated with either the BS^3 solution or vehicle (1 mL below insert and 0.5 mL above) using gentle agitation for 15 min at 4 °C. After incubation, inserts were again placed over ice, and then washed twice with a 1 M Tris-ACSF solution to quench the cross-linking reaction. Slices were then gently scraped from inserts (9–12 slices/condition) and transferred to 250 µL of non-ionising lysis buffer supplemented with a protease inhibitor cocktail. Following manual homogenisation over ice, lysates were centrifuged at 1000 × g for 10 min (4 °C), the supernatant protein concentration determined with a BioRad DC protein assay kit, and samples either used for SDS-PAGE or stored at –80 °C.

SDS-PAGE and Immunoblotting

Sample homogenates were loaded in duplicate or triplicate (20 µg protein/lane), resolved using SDS-PAGE, and transferred to PVDF membranes (NEN Life Science Products, Boston, MA, USA). Notably, cross-linking of surface proteins with BS^3 creates a large complex that does not migrate during SDS-PAGE, and only intracellular proteins from these samples would be expected to have been resolved. Membranes were blocked for 1 h with 5% skim milk powder (w/v) prepared in TBST [20 mM Tris, 140 mM NaCl, 0.1% Tween-20 (v/v), pH 7.6], and immunoblotting performed by overnight incubation (4 °C) with anti-IR β-subunit (1:1 000, rabbit polyclonal, sc-711; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α7 nAChR (1:500, rabbit polyclonal, ab23832; abcam, Cambridge, MA, USA), or anti-actin (1:5 000, rabbit polyclonal, A2066) antibodies as appropriate. Membranes were washed and placed into the appropriate HRP-linked anti-mouse/rabbit secondary antibody (1:5 000 for all except actin, which was 1:10 000) for 1 h. All antibody solutions were prepared in blocking buffer. Membranes were then treated with an ECL solution (Amersham Biosciences; Baie d'Urfé, PQ, Canada) and exposed to imaging film (Kodak; Amersham Biosciences). Developed films were scanned and the relative density of each band of interest, from within the linear range of exposures, was measured, background-subtracted, and, where appropriate, normalised to actin. As appropriate, membranes were placed into stripping buffer (ReBlot Plus; Millipore) for 15–20 min at room temperature with constant agitation before being re-probed.

Statistical analysis

Data are expressed as mean and standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (San Diego, CA, U.S.A.). Comparison of means was completed with the Student's t test, and significance was set at $p < 0.05$.

RESULTS

Insulin labelling of OHSCs remains stable over time in culture

Fluorescently labelled insulin (INS-FITC) has been used to examine insulin binding within dissociated neurones (Mielke *et al.* 2006), and we modified the technique for use in cultured slices (Figure 1A). Cultured slices treated with INS-FITC displayed a concentration-dependent increase in fluorescence [e.g., 10 nm INS-FITC: 160.9 ± 31.0 arbitrary fluorescence units (AFUs); 100 nM INS-FITC: 579.4 ± 57.1 AFUs; n=7–11 separate cultures; Figure 1B]. Notably, our previous work with dissociated neurones used 100 nM INS-FITC, so this concentration was used for subsequent experiments in the present study. In addition, when slices were incubated with 100 nM INS-FITC in the presence of an equal, or substantially greater, amount of unlabelled insulin (uINS), the degree of labelling was significantly reduced (% 0 nM uINS AFUs: 100 nM uINS, 66.8 ± 7.7%; 5 000 nM uINS, 42.5 ± 14.0%; n=6–8 separate cultures; Figure 1C).

Insulin is believed to play an important neurotrophic role; for example, the hormone is required for the maintenance of neurones (Snyder & Kim 1979; Snyder & Kim 1980; Aizenman *et al.* 1986; Aizenman & de Vellis 1987) and glia (Morrison & de Vellis 1981) in defined media, and influences macromolecule synthesis in both neuronal (Raizada *et al.* 1980; Yang & Fellows 1980) and glial (Clarke *et al.* 1985) cultures. As a result, insulin binding would be expected to remain steady over time in culture; however, several rodent studies have noted the progressive decline of neural insulin binding with time (Kappy & Raizada 1982; Lowe Jr *et al.* 1986; Bren-

nan Jr 1988; Pomerance *et al.* 1988; Marks & Eastman 1990). To determine whether insulin binding might change during a typical three week culture period, INS-FITC labelling was assessed at two points. The degree of INS-FITC binding was observed to remain stable (6–8 DIV, 579.4 ± 57.1 AFUs; 19–22 DIV, 788.1 ± 157.1 AFUs; n=5–9 separate cultures; Figure 2).

Levels of the IR β-subunit protein decrease with time in culture

In general, insulin receptors are synthesised as polypeptides that undergo dimerisation and proteolytic processing, which leads to a heterotetramer composed of two extracellularly positioned α-subunits and two membrane-spanning β-subunits remains (Olefsky 1990; Lee & Pilch 1994). The mature IR is a multi-domain protein with two major functional components: a ligand-binding region composed of the α-subunits, and a signal transduction domain attributable to residues within the β-subunits. The neural IR bears considerable structural similarity to its peripheral counterpart; however, both constituent subunits of the neural variant do possess a slightly greater molecular weight, which has been shown to be attributable to post-translational modification (Mielke & Wang 2011).

Over development, proteins expressed within OHSCs display a varied profile; for example, whilst levels of glutamate receptor subunit proteins are thought to remain stable (Bahr *et al.* 1995), levels of certain synaptic proteins increase (Buckby *et al.* 2004) and levels of certain cytoskeletal proteins decline (Mielke *et al.* 2005a). To determine the pattern best describing the IR β-subunit during maintenance in culture, slices from the same preparation were harvested over four weeks, and the homogenates subjected to quantitative immunoblotting. Levels of the β-subunit declined precipitously during the first three weeks in culture, and appeared to reach a plateau around 28 DIV (percent of 0 DIV levels ± SEM: 34.0 ± 7.0%, n=5 separate cultures, Figure 3). Notably, the decline in protein expression was not a generalised phenomenon, for levels of the nicotinic acetylcholine receptor α7 subunit displayed an opposite trend.

The majority of IR β-subunits remain located at the cell surface over time in culture

For receptors that transduce extracellular signals at the plasma membrane, the degree of expression at the cell surface is a critical determinant of their functionality

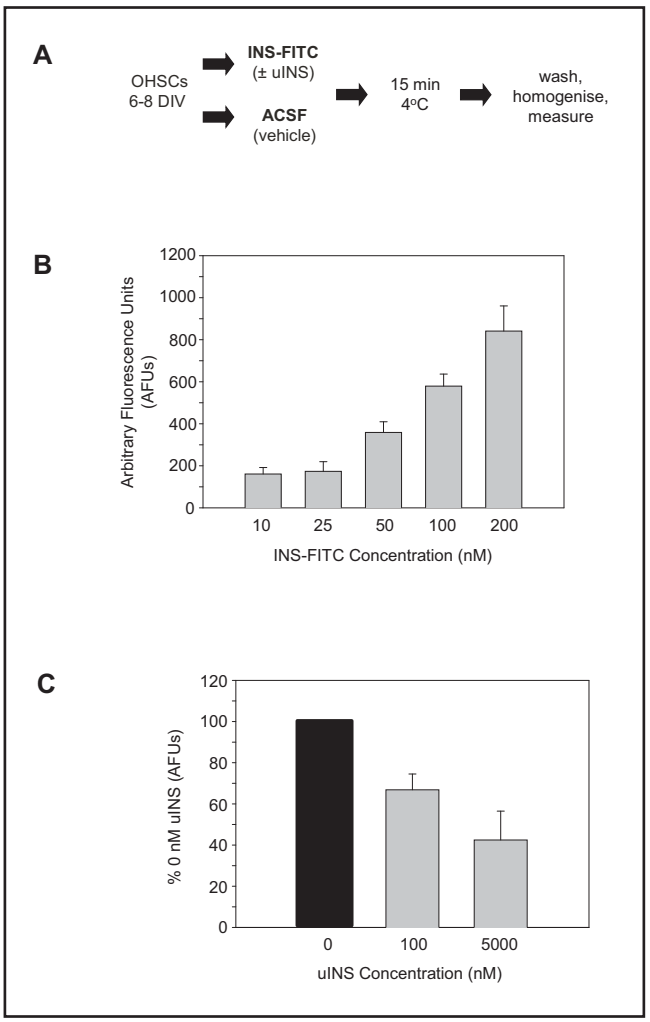

Figure 1 A fluorescence based assay to assess insulin binding in OHSCs. (A) The schematic outlines the design of experiments completed to characterise the INS-FITC assay. Since the experiments were completed with a physiological buffer, the assumption was made that the labelling reflected interaction with IRs positioned at the plasma membrane. (B) Increasing concentrations of INS-FITC lead to greater levels of fluorescence. The bars represent the mean ± SEM of 7–11 separate cultures. (C) Co-incubation of 100 nM INS-FITC with two concentrations of unlabelled insulin caused a reduction in fluorescence (% 0 nM uINS AFUs: 100 nM uINS, 66.8 ± 7.7%; 5 000 nM uINS, 42.5 ± 14.0%). The bars present the mean ± SEM of data from 6–8 separate cultures.

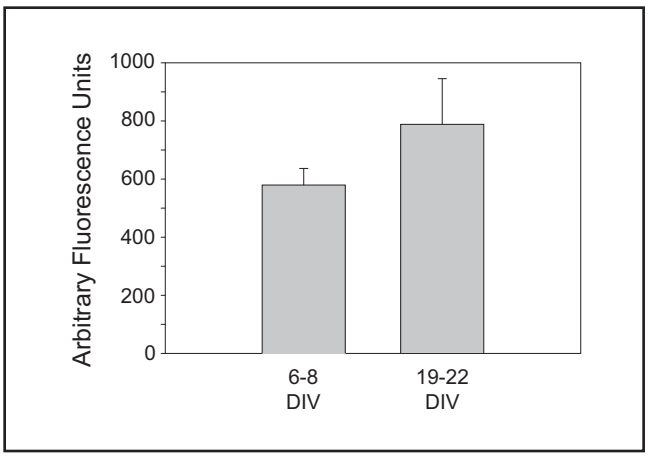

Figure 2 Insulin labelling remains stable over time in culture. OHSCs were maintained for approximately either one or three weeks prior to application of 100 nM INS-FITC. Length of time in culture did not lead to a statistically significant change in the amount of fluorescence detected (6–8 DIV, 579.4 ± 57.1 AFUs; 19–22 DIV, 788.1 ± 157.1 AFUs). The bars represent the mean ± SEM of 5–9 separate cultures.

(Wang *et al.* 2004; Kennedy & Ehlers 2006). To determine the relative proportion of IRs present at the cellular surface in OHSCs, and whether this distribution changed during time in culture, a membrane impermeable protein cross-linker was used (Mielke & Mealing 2009; Figure 4A). Following a week in culture, a majority of the β-subunits was able to be removed from the total cellular pool by cross-linking surface proteins (internal pool: 44.9 ± 8.7% of total, n=4 separate cultures, Figure 4B). When the same experiments were performed with OHSCs at approximately 21 DIV, a greater, albeit not statistically so, proportion of the total cellular pool was removed (internal pool: 26.9 ± 4.3% of total, n=4 separate cultures, Figure 4B).

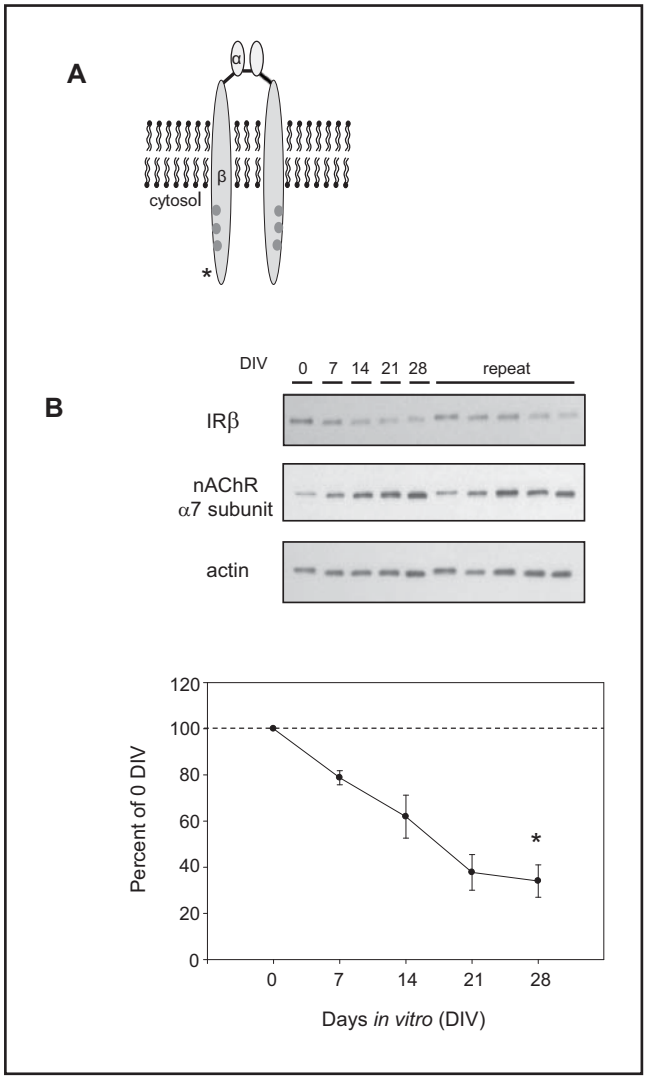

Figure 3 Protein levels of the IR β-subunit decrease over time in culture. (A) The schematic illustrates the heterotetrameric nature of the insulin receptor. The ligand binding region is formed by the extracellular α-subunits, which are attached to each other and the transmembranous β–subunits with disulphide bonds. Circles within the β–subunits denote regulatory loop tyrosine phosphorylation sites, whilst the asterisk highlights the c-terminal domain, which is the region of the epitope used for antibody labelling. (B) Representative films display samples harvested sequentially from the same preparation and probed for the IR β-subunit, nicotinic acetylcholine receptor α7 subunit, and an actin epitope. The graph presents densitometric analysis of blots wherein the background subtracted values for the target bands were normalised to actin and compared to the value at 0 DIV for each preparation. The circles represent the mean ± SEM of five separate cultures. *$p<0.05$, comparison with 7 DIV, Student's t-test.

DISCUSSION

To better understand the brain's insulin receptor, the present study examined the receptor within cultured hippocampal slices, which are a well-characterised model of a structure considered integral for the creation and processing of memory. We assessed three key IR features (ligand binding, the level of expressed β-subunit protein, and the surface density of the β-subunit) to see whether the values changed as a function of time in culture. We observed a prominent time-dependent decline in IR β-subunit protein expression

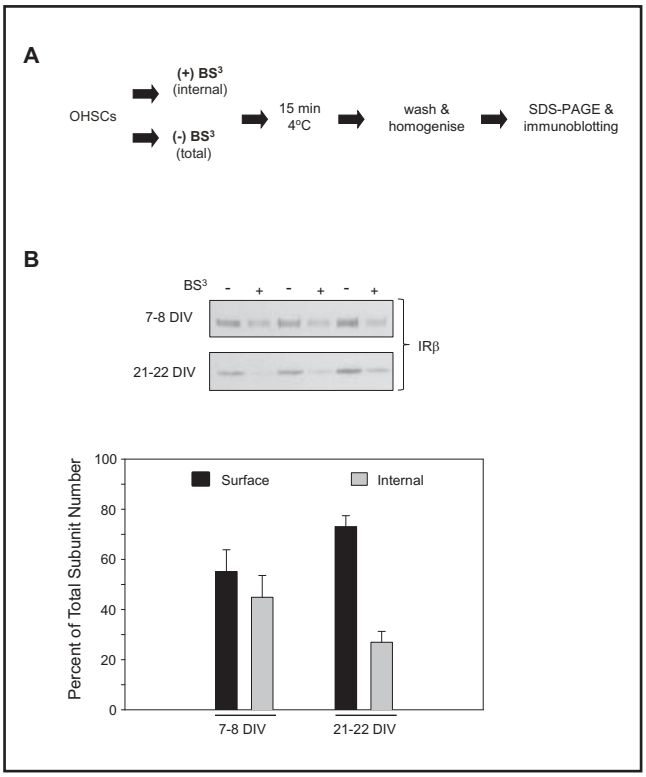

Figure 4 Most IR β-subunits are found at the surface of cells in OHSCs. (A) The schematic outlines the protocol used to cross-link surface proteins with BS^3, which created a complex too large to be resolved with SDS-PAGE. (B) The representative films display triplicate samples prepared from slices maintained for different lengths of time in culture, and probed for the IR β-subunit. The graph presents densitometric analysis of blots wherein values for the (–) BS^3 condition (i.e., internal pool) were taken as a percentage of the values for the respective total amounts of IR β-subunit protein. Surface expression values were determined by calculating the difference between total and internal levels of the protein. The bars present the mean ± SEM of data from four separate cultures.

that affected neither insulin binding, nor the proportion of the subunit present at the plasma membrane.

Potau *et al.* (1991) observed decreased insulin binding between synaptosomal membrane fractions prepared from the cerebral cortex of pre-term foetuses (i.e., <30 weeks of gestation) and newborns. In addition, a number of rodent studies have found that insulin binding to membrane preparations from various brain regions decreased sharply between the early neonatal period and adulthood (Kappy & Raizada 1982; Lowe Jr *et al.* 1986; Brennan Jr 1988; Pomerance *et al.* 1988; Marks & Eastman 1990). As a result, the degree of neural insulin binding, particularly in the earliest part of the life span, would seem to be quite dynamic. Given that rat brain development at the time of OHSC preparation (i.e., post-natal day 7) is regarded as equivalent to the stage that would be seen in a near term human brain (McIlwain & Bachelard 1971), we were curious about the stability of insulin binding during OHSC maintenance. Importantly, no statistically significant changes in labelling were observed in our experiments.

Since our insulin binding experiments did not reveal a change in labelling, we anticipated that expression of the IR β-subunit protein would also remain stable; however, levels of the protein declined dramatically. Whilst IR mRNA levels in rat brain peak around birth and decline thereafter (Baron-Van Evercooren *et al.* 1991), which agrees with our observation, the possibility did exist that a general reduction in protein expression brought about by maintenance of the tissue *ex vivo* was responsible. Importantly, the changes observed in the β-subunit over the course of four weeks in culture were not mirrored by the pattern of expression seen for a nicotinic receptor subunit during the same time frame, which suggests that the decline was not an artifact of the culturing process. As well, earlier studies using OHSCs have shown that levels of various cytoskeletal and synaptic proteins either remain stable, or increase with time in culture (Bahr *et al.* 1995; Buckby *et al.* 2004; Mielke *et al.* 2005a).

To reconcile our binding and protein expression data, we considered the possibility that a change may have occurred in the cellular distribution of the β-subunit. Notably, the functional profile of ion-channel receptors has been shown to be greatly influenced by their surface expression (Wang *et al.* 2004; Kennedy & Ehlers 2006); as a result, we thought that the stable insulin labelling may have been attributable to enhanced IR surface expression compensating for declining subunit levels. Since we found that approximately half of all β-subunits were intra-cellularly localised around post-natal day 7, a substantial internal pool of receptors, which could be re-directed to the surface, seemed to exist. However, our hypothesis was not supported, for the relative distribution of the β-subunit did not change to a statistically appreciable degree.

Previous work that used either rat liver preparations (Lopez & Desbuquois 1983), or isolated adipose cells (Hedo & Simpson 1984) revealed that the majority of insulin receptors are positioned at the cell surface; however, our present study, and earlier work completed with dissociated neurones (Mielke *et al.* 2006), suggest that a large internal pool of the receptor does exist. One explanation for the contrasting distribution patterns of the IR may be the different physiological roles that insulin is thought to play in neural versus non-neural tissue. Whilst the predominant outcome seen following insulin stimulation of canonical tissue is an increase in glucose uptake, within brain tissue insulin activates a number of other cellular responses; in particular, modulation of the surface expression of many ion-channel receptors that underlie neuronal communication (Wan *et al.* 1997; Skeberdis *et al.* 2001; Ahmadian *et al.* 2004).

Neuronal transmission is a very dynamic process that requires synapses to have the ability to rapidly modify signal strength. The retention of an internal pool of IRs might provide neurones with a reserve population of the receptor that can be upregulated, and, in so doing, provide a mechanism whereby the surface expression of receptors directly tied into neuronal communication can be modulated. The high priority that neurones place on maintaining the fidelity of synaptic communication would likely translate into a desire to also maintain related systems that assist in the process. As a result, despite a declining number of β-subunits, cells within OHSCs may actively work to keep the relative cellular distribution of the IR similar over time.

Considered together, our results suggest that, during typical OHSC maintenance conditions, both insulin labelling and the proportion of the β-subunit positioned at the cell surface are relatively stable, despite a reduction in overall levels of the subunit. The neurotrophic role played by insulin (Snyder & Kim 1979; Raizada *et al.* 1980; Snyder & Kim 1980; Yang & Fellows 1980; Morrison & de Vellis 1981; Clarke *et al.* 1985; Aizenman *et al.* 1986; Aizenman & de Vellis 1987), together with the influence that the hormone has upon receptors important for neuronal communication (Wan *et al.* 1997; Skeberdis *et al.* 2001; Ahmadian *et al.* 2004; Mielke & Wang 2011), would seem to provide good reasons for neurones to maintain a set surface density of the IR. Although the mechanism whereby insulin receptor function is maintained remains unclear, one possible explanation may be found in the way in which the cell manipulates the distribution of available receptors. Many details remain to be added to complete our understanding of the neural IR, but the present study does suggest that OHSCs provide a valuable model system to study the unique neural IR subtype.

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