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Antisense delivery and protein knockdown within the intact central nervous system

Michael Cronin [1], Patrick N Anderson [1], Colin R Green [2] and David L Becker [1]

[1] Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, United Kingdom, [2] Department of Ophthalmology, University of Auckland Medical School, Auckland, New Zealand

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1. ABSTRACT

The ability to down regulate the expression of a specific protein within the intact central nervous system (CNS) is highly desirable from both a research and therapeutic perspective. Antisense has the potential to do this. However, problems of invasive antisense delivery methods and short half life of remain problematic. We overcome this by using Pluronic gel to provide a sustained delivery antisense oligodeoxynucleotides (ODN's) to the intact central nervous system and achieving rapid penetration throughout the spinal cord in 2-3 hours and significant knockdown of our target protein connexin 43 (Cx43) in 4-8 hours (recovering at 48 - 72 hours). Interestingly CY3-siRNA probes could not be detected penetrating the intact CNS and no knockdown the Cx43 was found. This approach with conventional ODNs could provide a faster and cheaper alternative to knockout mice in the investigation of the functions of specific proteins within the CNS and may also have therapeutic implications for drug discovery and development.

2. INTRODUCTION

Antisense approaches represent an elegant and simple way to quickly screen the function of new genes in the CNS as they rapidly come to light in the post genomic era. They have distinct advantages over knock out mice in terms of time and expense and do not have the complications of the effects of genetic background, compensation by other genes and potential developmental defects. Applied to a normal adult central nervous system antisense can used to find out what a particular protein actually is used for or to test for targets in drug development. Indeed, if effective the antisense may be a prime candidate for a drug itself.

However, there remain some significant hurdles in the use of antisense in the CNS of which stability and means of delivery are perhaps the most important to overcome (1.). To date intravenous delivery of antisense oligodeoxynucleotides (ODN's) across the blood brain barrier (BBB) has been ineffective (2,3.) and until now

most effective delivery of antisense to the CNS has been by direct physical injection or infusion into the CNS or ventricles. Whilst this has proved effective it causes damage and thereby reduces the appeal of antisense as a potential therapeutic agent. Once in the CNS unmodified antisense probes are unstable and are rapidly broken down by the ubiquitous nucleases. Early modifications of the antisense chemistry, designed to prolong their life, produced some new problems associated with toxicity, non specific binding effects and occasional reports of eliciting immune response (1.). However, progress in understanding antisense technologies has overcome many of these early problems and we now have a variety of highly effective antisense approaches available to us. Target protein knock-down can be achieved with conventional antisense oligonucleotides, ribozymes, morpholoinos (4.) or with the more recently discovered short interfering RNA (siRNA) technology (5,6). SiRNA has been heralded as the new generation of antisense technology, providing a more effective and longer lasting knockdown. It has been shown to be a very effective approach in plants and invertebrates and more recently in mammalian cells. Indeed, siRNA has also been shown to be able to down regulate CNS proteins *in vitro*, (7,8). However, the application of siRNA *in vivo* has been limited, often requiring transfection agents or viral delivery to introduce the siRNA into target cells.

The challenge remains to deliver these antisense technologies to the CNS in a non-invasive manor. Here we describe the use of Pluronic gel as a delivery agent for unmodified antisense to the intact CNS. We compare its efficacy for conventional unmodified antisense ODN's and siRNA in terms of the time course of penetration throughout the intact spinal cord, lifespan of the agent and the knock-down and recovery of the target protein, connexin 43 (Cx43). Our target protein, Cx43, is found in astrocytes and some neurons throughout the CNS and its expression is greatly increased in cases of CNS damage, disease or inflammation (9-13.). Several reports have indicated that communication through this gap junction channel may have negative effects in the distressed CNS making it a potential target for therapeutic investigations (14-16.). Lack of pharmacological agents that act as specific blockers of connexins make this antisense approach a valuable tool in the study of connexins.

3. MATERIALS AND METHODS

3.1. Antisense probes and application

Cx43 antisense ODNs (Sigma Genosys) were unmodified 30mers 5'-GTAATTGCGGCAGGAGGAATTGTTTCTGTC-3'. the controls for which were the sense version, a scrambled version or gel alone (17,18,). For the penetration studies a CY3 tag was added to the ODN. CY3 and CY5 tags were used for donor and acceptor FRET pairs tagged to alternate ends of the ODNs. ODNs were generally prepared in pluronic gel to a 1μM concentration based on the molecular weight of the nucleotides alone. The siRNA was designed to target the transcription initiation site of Cx43: 5'-CAGUCUGCCUUUCGCUGUA-TT-3'19 pecific RNA bases plus 2 DNA base overhangs 3'-GUCAGACGGAAAGCGACAU-AA-5. The 21mer pairs were annealed [briefly, siRNA pairs were mixed in annealing buffer (50mM Tris, pH 7.5-8, 100mM NaCl in DEPC-treated water), incubated for 1min at 90 - 95 ^{0}C and allowed to cool slowly to room temperature. Once annealed duplex siRNA can be safely stored in annealing buffer at -20 ^{0}C. For penetration studies a CY3 tag was added to the siRNA. Final concentrations of siRNA in Pluronic gel used in the experiments described were 0.5, 1.0 and 10μM. (siRNA was supplied by Eurogentec).

Male Sprague-Dawley rats (200-250g) were anaesthetised with 2% halothane and nitrous oxide: oxygen (2:1) and a partial laminectomy was performed to expose C3 to C5. In some pilot experiments the dura was opened with a microscissors (n=5), prior to CY3-ODN application whilst in all others it was left intact after it was found to be no significant barrier to ODN penetration. A 250μl aliquot of 30% Pluronic gel F-127 (Sigma) in DEPC water was used to deliver the various antisense probes. Pluronic gel is liquid at 0-4 ^{0}C, but sets rapidly when warmed by living tissue at higher temperatures (17.). The rapid setting of the gel necessitates swift application and the use of instruments that are kept on ice in order to keep them cool. Following application of the gel to the exposed spinal cord, overlying musculature was sutured and the surgical site closed with suture clips.

3.2. Penetration studies – ODNs and siRNA

Penetration of CY3-ODNs and CY3 siRNA into the intact spinal cord was examined at a series of time points; 2, (N=3) 4,(N=3), 8,(N=3), 24,(N=3), 48,(N=3) and 72,(N=3) hours post application for antisense ODN probes and 2, (N=3), 8, (N=3), 24, (N=3), 48, (n=3) and 72, (N=3) hours for siRNA. At these time points anaesthesia was induced with halothane and animals were given an overdose of sodium pentobarbitone by IP injection. The cervical spinal cords (C3-C5) were quickly dissected out and fresh frozen in O.C.T. (TissueTek) on dry ice. Serial transverse cryosections of 20μm were collected on poly-L-Lysine coated glass slides and stored at -20^{o}C. Sections were post fixed in 4% paraformaldehyde for 1min and washed in PBS prior to counterstaining their nuclei with Hoechst 33342 (0.2μg/ml) and mounting in Citifluor (Cantebury Kent). Sections were viewed using a X63 1.32 Na objective on a Leica TCS SP confocal laser scanning microscope. Hoechst labelled nuclei were excited with the 351 / 363 nm UV lines and viewed in the blue range whilst Cy-3 tagged ODNs were sequentially excited using the 563nm laser line and viewed in the red range. The dura and four key regions of each spinal cord section were examined DWM, DGM and VGM as shown in Figure 1a. High resolution images were captured from each of the regions using identical parameters.

3.3 . FRET studies

Antisense ODNs were generated with FRET pair fluorophores CY3 and CY5 tagged to opposite ends of the ODN. These were applied to the spinal cord at 10uM concentration using standard procedures (N=4). At 1hr, 3hr and 6 hr time points the cords were harvested,

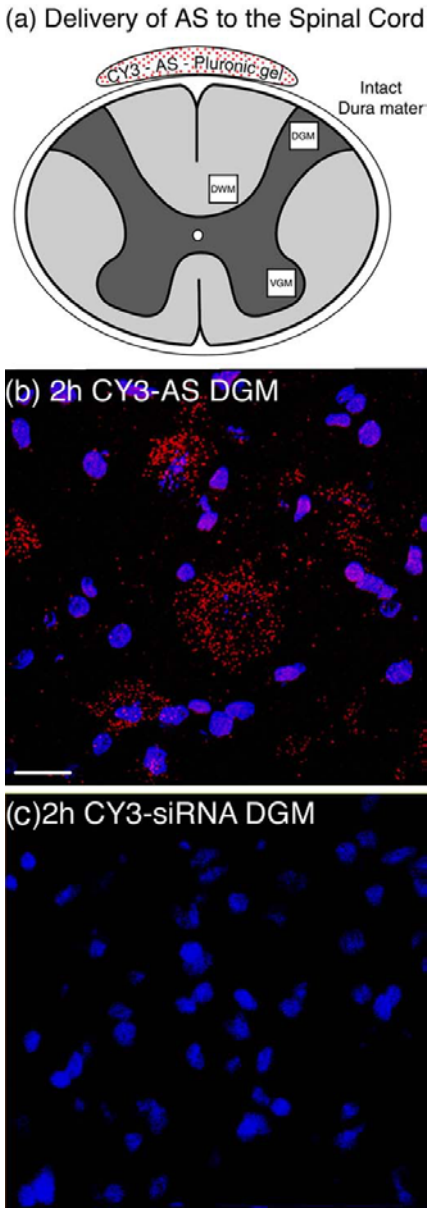

Figure 1. Delivery of Antisense to the intact Spinal Cord. (a) Schematic diagram showing the delivery of antisense in Pluronic gel to the cervical spinal cord with the dura mater intact. The regions of the spinal cord dorsal white matter (DWM), dorsal grey matter (DGM) and ventral grey matter (VGM), that were examined for CY3-antisense penetration and Connexin 43 protein immunostaining are highlighted in the diagram. These regions have the most consistent and homogenious Cx43 staining within the spinal cord. (b) Conventional CY3-ODN penetration into the DGM at two hours after Pluronic gel application to the spinal cord. (c) DGM two hours after CY3-siRNA was applied to the spinal cord in Pluronic gel. No CY3 fluorescence was detected at any time point examined (2-72 hours). Nuclei are counterstained with Hoechst 33342 (blue). Scale bar 25μm.

transverse sections taken and transferred in gassed Krebs maintained at 37^{o}C for examination on a Leica SP2AOBS confocal microscope with an incubation chamber dedicated to live cell imaging. Labelled cells were imaged using a x40 0.8NA Hcx water immersion objective. In some instances the 543nm laser line was used to excite CY3 whilst emitted fluorescence was picked up simultaneously in both the red (coloured red) and far red spectra (later coloured blue). Alternatively a lambda scan was carried out, stimulating CY3 with the 543nm laser line but detecting the emitted fluorescence in a series of 50 X 5nm bins from 548nm - 727 nm. The lambda scan was later analysed for fluorescence intensity in individual cells at progressive wavelengths over the red and far red spectrum.

3.4. Knockdown and recovery studies

Immunohistochemistry for ODN applications at 2, (N=5), 4,(N=5), 8,(N=4), 24,(N=5), 48,(N=5) and 72,(N=4) hour survival times, and siRNA and 2, (N=3), 8, (N=3), 24, (N=3), 48, (n=3) and 72, (N=3) hours survival, spinal cords were collected and sectioned as above. Tissue sections were washed in PBS, blocked with 0.1M lysine / 0.05% Triton X-100 (Sigma) for 1 hour, and incubated overnight at 4 ^{0}C in primary antibody diluted in blocking solution. Cx43 was detected using a monoclonal antibody GAP1 (19.) at 1:200 dilution. Following primary incubation the slides were washed three times five minutes in PBS before incubation in anti-mouse Alexa 488 (Molecular Probes) secondary antibodies at 1:200 in the dark at 37^{0}C for one hour. Slides were then washed three times five minutes in PBS before mounting in Citifluor (Cantebury, Kent). Hoechst 33342 (Sigma) counter stain was included in the first of the final washes in order to distinguish cell nuclei in all sections. All processing steps were carried out in humidified incubation chambers.

3.5 Analysis of Cx43 knockdown and recovery

Immunostained preparations viewed using a X63 1.32 NA objective on a Leica TCS SP on a confocal microscope using the 488 nm excitation laser line and collecting signal in the green spectrum. The power of the laser, pinhole, offset and gain were optimised and kept constant between preparations. Semi-quantitative analysis of Cx43 protein knockdown and recovery was carried out on two animals at each time point, all processed for immunostaining and anlaylsed by confocal microscopy in one session in order to reduce variation. Three regions were analysed, DWM, DGM and VGM in six sections from each animal at each time point. All imaging parameters were kept constant during collection and images were stored digitally and exported to Image J for analysis or Adobe Photoshop for illustration. Image analysis of connexin expression was carried out using standard procedures from our lab (20.). In brief, all images were subjected to a standard threshold to create a binary image. An Image J macro was then used to count all of the connexin plaques and positive pixels, the data from which was exported into excel for graphing and statistical analysis. Spearman Rank Correlation analysis was used to assess the knockdown and recovery of Cx43 protein levels.

4. RESULTS

4.1. Antisense penetration

We have used CY3 tagged antisense, in the form of unmodified ODNs (CY3-ODNs) and siRNA (CY3-siRNA) to examine the penetration of these probes into the intact spinal cord, with the dura intact or removed, using Pluronic gel as a delivery agent (Figure 1). We found a striking difference between the penetration of ODNs and siRNA into the CNS. Whilst single stranded ODNs entered the CNS rapidly the double stranded siRNA did not appear to enter the dura or CNS at all as no fluorescence from CY3-siRNA could be detected in these tissues at any time point examined 2-72 hours.

Interestingly the dura did not seem to present a significant barrier to single stranded antisense ODN penetration and no difference in CY3-ODN penetration rates and extent between subdural and epidural application was observed. Using high resolution confocal microscopy, CY3-ODNs could be detected as punctate vesicles in the somata in large cells throughout the spinal cord within two hours of application. At this stage CY3-ODN labeling was particularly pronounced in the dorsal and ventral grey matter (Figure 1b). This represents a distance of 300-400 μm that the ODN's have penetrated in 2 hours. Within 4 hours virtually all cells of the spinal cord, large and small, contain the CY3ODNs.

The CY3 signal can first be detected in the larger cells (presumptive neurons) of the grey matter and the signals were always strongest in these cells. The smaller cells (presumptive glial) were more slow to accumulate the CY3 and were never as brightly labelled as the large cells. The CY3 signal could still be detected throughout the spinal cord at 48hrs and 72hrs but this is not likely to reflect the presence of intact antisense as the half life of the unmodified ODNs is a matter of hours.

4.2. Fluorescence Resonance Energy Transfer "FRET" – To monitor Antisense ODN breakdown within the spinal cord

Use of FRET combination fluorescent probes, tagged on opposite ends of the asODN, is able to demonstrate the breakdown of the probes within the living tissue by nucleases. Whilst breakdown of unmodified ODNs is fairly rapid, when in contact with nucleases, its continuous replenishment from the gel ensures its sustained action. Visualising the red and far red spectra emitted after stimulation with only the 543nm laser line gave an indication of the amount of FRET taking place in the living tissue and therefore the extent of breakdown of the ODNs (Figure 2a,b). Examination of transverse sections of spinal cord at one, three and six hours after application revealed the extent of breakdown of ODNs as indicated by relative amounts of red fluorescence of CY3 which was not undergoing FRET (due to breakdown) and far red from FRET (indicating intact ODNs). At one hour most of the detectable fluorescence was in the far red range with only a little in the red range. By three hours significantly more fluorescence was visible due to increased penetration of the ODNs into the spinal cord. At three hours a lot of the fluorescence was red, however, there was also considerable far red fluorescence at this time indicating that FRET was still taking place and a significant proportion of ODNs were still intact. By six hours the majority of the signal was in the red range indicating that there had been a lot of breakdown of the ODNs. At this time point the larger cells showed more FRET than the smaller ones, an effect shown in the lambda scan analysis of fluorescence intensity at progressive wavelengths illustrated for three different size cells in Figure 2c.

4.3. Cx43 protein knockdown and recovery

Using immunolabelling for Cx43 protein we were able to demonstrate the time course of knockdown and recovery of expression of our target protein, Cx43, in the spinal cord following a single application antisense ODNs in Pluronic gel. Connexin 43 protein knockdown in the CNS occurs very quickly after application of the antisense ODNs. However, no reduction in staining was seen, at any time point, after application of siRNA. This is likely to be due to the fact that the siRNA does not appear to enter the spinal cord when delivered in this way. We know that this siRNA is effective in reducing Cx43 protein expression if transfection reagents are used to get it into cells (Frank and Becker, unpublished observations).

The heterogeneous nature of the cells of the spinal cord is reflected in the heterogeneous staining for Cx43 protein and its different turnover rates. Figure 1a indicates regions of Dorsal White Matter (DWM), Dorsal Grey Matter (DGM) and Ventral Grey Matter (VGM) spinal cord where staining patterns were most uniform and consistent (21.). These areas were imaged using standardized confocal microscopy in order to provide a semi-quantitative analysis of the Cx43 protein staining, its knockdown and recovery. Graphs of total numbers of Cx43 positive pixels in images from each region at all time points (shown in Figure 3a) provide an indication of the Cx43 protein expression profiles. The DWM was the first area to show a marked decrease of Cx43 staining within two hours of ASODN application, becoming almost undetectable at four, eight and 24 hours and only recovering at 72 hours (Figure 3a). Despite the variation of staining associated with different cell types of the spinal cord, within four-eight hours a significant decrease of Cx43 immunostaining signal is evident throughout the cord. Cx43 staining profiles in the VGM (Figure 3c) were very similar to the DWM in terms of knockdown and recovery whilst the DGM profile showed a sharper knockdown at eight hours with recovery evident at 24 hours and back to normal levels within 48 hours (Figure 3b). The punctate nature of the Cx43 staining and its sharp profile of changes over the time course examined in the DGM is illustrated in figure 2 d. At the peak of knockdown in excess of 90%, of normal Cx43 staining had vanished in all three areas examined. As Cx43 protein knockdown progressed plaques became smaller and fewer in number before disappearing almost entirely in some cases. Recovery of staining was the reverse of this process, small plaques starting to appear then gaining in size by 72 hours.

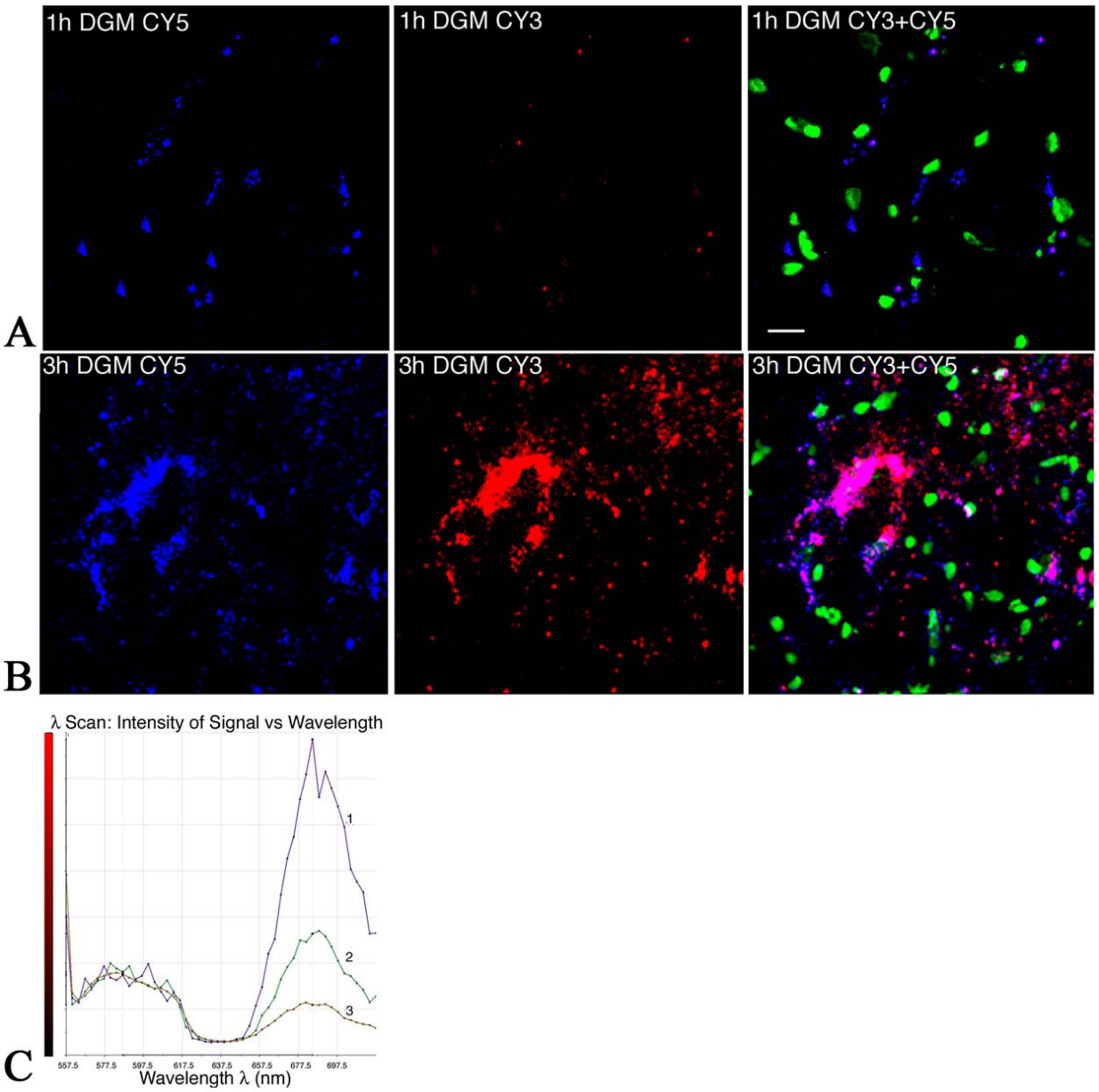

Figure 2. FRET analysis of antisense ODN stability in the Spinal Cord. (a,b) Fluorescence resonance energy transfer (FRET) is detectable when ODNs are intact and fluorescence is emitted in the far red range (coloured blue). FRET does not occur when the ODNs are degraded and fluorescence is then emitted in the red range (coloured red). Nuclei are counterstained with Hoechst 33342 (green). Direct visualization of intact and degraded ODNs in the DGM at 1 hour (a) and 3 hours (b) after application to the intact spinal cord using Pluronic gel. (c) Confocal microscope lambda (λ) scan plots showing the relative intensity of emitted light at wavelengths between 548nm and 727nm within 3 different size cells from the spinal cord three hours post-application. Large cells (1) and intermediate sized cells (2) showed more FRET activity (peak emission intensity in the far-red range) than in smaller cells (3) where peak emission intensity is in the red range.

5. DISCUSION

5.1. Penetration: ODNs vs siRNA

We have observed a complete contrast in the ability of double stranded siRNA and single stranded ODNs to penetrate the intact CNS. Pluronic gel delivered double stranded siRNA does not appear to enter the cells of the dura or the CNS whereas the single stranded ODNs are rapidly incorporated into all cells of the spinal cord. Uptake of the CY3-ODNs appears to be fastest in the large neurones of the dorsal and ventral grey matter, which might be expected given the spread of their dendritic trees within the spinal cord. Such rapid uptake of ODNs has been reported previously in vivo following physical injection into the CNS or ventricles (22.) but to date delivery of antisense ODNs across the blood brain barrier following IV

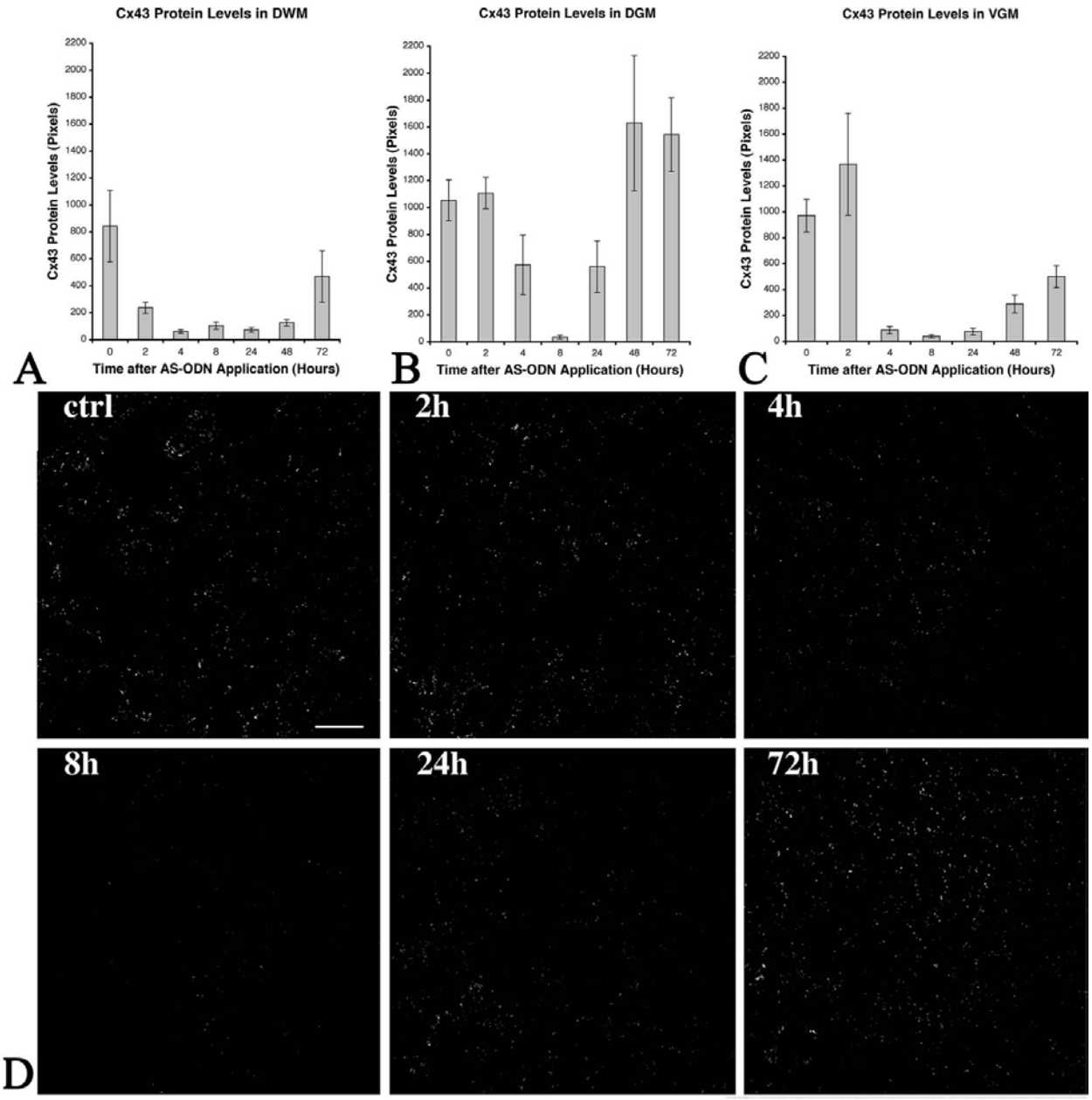

Figure 3. Knock-down and recovery of Cx43 protein in Spinal Cord. Semi-quantitative analysis of the knock-down and recovery Cx43 immunostaining in (a) DWM, (b) DGM, and (c) VGM, over a 72 hour period following delivery of 1μM antisense ODN in Pluronic gel to the intact spinal cord. A significant knockdown of over 90% of Cx43 immunostaining was observed in all three areas within 4 to 8 hours, with recovery evident between 24 and 72 hours. (d) Confocal micrographs showing the punctate nature of Cx43 staining in spinal cord DGM and the changes observed following AS-ODN application. Median examples shown for control, 2h, 4h, 8h, 24h and 72h. (Scale bar 25μm).

administration has been ineffective with less than 1% of the delivered ODNs being detected in the CNS (2,3.). Delivery of siRNA appears to require transfection like procedures to penetrate cells in vivo and in vitro. This Pluronic gel delivery approach appears to be promising for conventional ODN delivery to the CNS but more work is required for the effective delivery of siRNA to the intact CNS.

5.2. Pluronic gel delivery

Pluronic gel is a non-toxic delivery agent that acts as a reservoir for the slow release of the unmodified ODNs allowing effective topical application, thereby overcoming two of the major problems in the use of antisense by producing a sustained targeted delivery. It also has the distinct advantage over previous approaches of direct injection into the CNS or ventricles of very high

concentrations of ODNS which damages the CNS as they are being administered means using a log scale lower concentration of ODNs. The mild surfactant action of pluronic gel is likely to aid rapid penetration of antisense ODNs into CNS tissue of the intact spinal cord although it is apparently of no assistance to the penetration of double stranded siRNA. The Pluronic gel sets in place when it comes into contact with the surface of the spinal cord, gradually releasing ODNs as it breaks down at its liquid interface. The concentration of the gel will determines the rate of breakdown (23, 24.) and hence release rate and length of action of the ODNs can be easily modified for the dynamics required for a particular target protein. Here we used a single topical application of 30% Pluronic gel containing 1μM antisense ODNs to produce this particular profile of Cx43 knockdown and recovery. The profile could be easily manipulated by changing the concentration of antisense (efficacy) and / or the concentration of the gel (rate of release) to increase or decrease the period of delivery and knockdown. It should also be possible to make less invasive epidural injections of the gel or infusions of very dilute gel via osmotic minipump in order to provide delivery of ODNs.

The sustained delivery that the Pluronic gel provides allows the use of very low concentrations of ODNs and this is essential when using unmodified ODNs that are extremely susceptible to breakdown by nucleases. The FRET studies at one hour post application, show that as the ODNs enter the cells there is some evidence of their breakdown beginning. By three hours CNS cells have accumulated considerable amounts of ODNs, and whilst a large part has been broken down there still remains a largely intact and active component. By six hours, using this set of delivery parameters, most of the unmodified ODNs have broken down and our antisense affect on Cx43mRNA will be nearing its end as we would expect. FRET analysis indicated that smaller cells contained more broken down ODNs compared to larger ones. This may reflect higher levels of nucleases in the smaller cells causing faster breakdown. More likely, this represents slower replacement of ODNs by the smaller cells compared to the larger ones which were the first to show signs of uptake and always contained more ODNs. Whilst the modification of antisense chemistries could prolong the life span of our ODNs this unmodified approach has the distinct advantage of avoiding some of the non specific effects that modified chemistries have previously been shown to cause such as non-specific binding and immune response (1). As the unmodified ODNs are broken down they are either utilized or excreted.

5.3. Knockdown and recovery of Cx43

Application of Cx43 siRNA to the intact spinal cord failed to produce a reduction in immunostaining for this protein at any time point examined from 2 – 72 hours. Had there been any effect on expression we would have expected to detect it within this time window. The lack of effect is most likely to be due to the lack of penetration of the siRNA into the spinal cord as we have previously found this Cx43 siRNA to be effective on a different *in vitro* system when used in conjunction with a transfection delivery agent (Frank & Becker unpublished observations).

Antisense ODNs can be detected throughout the spinal cord in 2-3 hours of application. Our target protein, Cx43, is normally rapidly turned over depending on cell type, sometimes in 1.5-2 hours (25.). Some down regulation of Cx43 staining can be seen in the DWM at 2 hours post treatment but there was little difference in the DGM and VGM at this stage, perhaps reflecting the different cellular composition. However, by 4-8 hours there was significant knockdown, in excess of 90%, in all three areas examined. The profile of knockdown and recovery was sharpest in the DGM and some recovery was evident at 24h hours and back to normal levels within 48 hours. However, the DWM and VGM took longer to recover and were still not at normal levels by 72 hours. FRET studies indicate that there is not likely to be much active antisense after 24 hours so the delayed recovery of Cx43 protein expression implies that the replacement rate in the DWM and VGM has much slower dynamics than the DGM. The DGM has previously been shown to contain one of the highest densities astrocytes and Cx43 expression (21.). The dynamics of turnover that we see are those to be expected given that astrocytes are the main Cx43 expressing cell type in the CNS and there appears to be a strong requirement for Cx43 protein in this region.

5.4. Advantages of antisense as an approach for studying a proteins function in the CNS

The use of antisense oligonucleotides to control protein expression is fast becoming a valuable tool in basic science and medicine because of its potential to rapidly generate potent and specific agents against target proteins. Indeed the first antisense drug vitravene obtained FDA approval in 1999 and several others are in late stages of clinical trials. Recent advances in genomic sequencing has revealed thousands of new targets that might benefit from antisense treatment and in response this area of antisense research is developing rapidly. Combining these new technologies with our novel antisense delivery mechanism increases the potential of antisense as a means of reducing the expression of target proteins in the CNS for research or therapeutic purposes.

Key advantages of using antisense are that it is relatively inexpensive and it is quick and easy to make and use in comparison to conventional transgenic gene knock outs which have associated problems of genetic background variability and potential complications with developmental defects or related gene compensation. With antisense ODNs, in combination with pluronic gel or osmotic minipump it is possible to provide direct and sustained delivery to target tissues. Using low concentrations of unmodified ODNs reducing chances of toxicity or non specific effects. The demonstration that antisense ODNs are able to rapidly enter the intact CNS and knock down the target protein opens up the potential to quickly and cheaply screen the function of a variety of proteins for their normal or pathological functions. In the case of the latter the ODNs themselves may prove to be a candidate drug for future development.

6. ACKNOWLEDGEMENT

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Send correspondance to: Dr David Becker, Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, United Kingdom, Tel: 020-7679-6610, Fax: 020-7679-6634, E-mail d.becker@ucl.ac.uk

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