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Successful use of axonal transport for drug delivery by synthetic molecular vehicles

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We report the use of axonal transport to achieve intraneural drug delivery. We constructed a novel tripartite complex of an axonal transport facilitator conjugated to a linker molecule bearing up to a hundred reversibly attached drug molecules. The complex efficiently enters nerve terminals after intramuscular or intradermal administration and travels within axonal processes to neuron cell bodies. The tripartite agent provided 100-fold amplification of saturable neural uptake events, delivering multiple drug molecules per complex. *In vivo,* analgesic drug delivery to systemic and to non-targeted neural tissues was greatly reduced compared to existing routes of administration, thus exemplifying the possibility of specific nerve root targeting and effectively increasing the potency of the candidate drug gabapentin 300-fold relative to oral administration.

INTRODUCTION

Pharmacological entrainment of axonal transport for the purpose of targeted drug deliver to selected sites is acknowledged as a highly desirable objective since it would allow for targeted antiviral, antineuropathic or regenerative treatments to segmentally selected ganglion or CNS cells. Methods using direct injection into nerves [1], introduction of modified neurotropic viruses [2], or delivery of neurotoxins such as tetanus toxin or ricin [3,4] have been explored but each pose barriers to routine clinical use. Phage display has been used to generate synthetic peptides to promote axonal transport [5], but it has not been clear how to exploit this.

Retrograde axonal transport of exogenous molecules from the periphery to CNS neuronal cell bodies is long established [6] and is one of the methodological bases for mapping neuroanatomical pathways [7-9]. The underlying physiology and biochemistry are increasingly well understood [10-14]. However, although axonal transport for drug delivery has been proposed previously [15-19], little or nothing is known about the major features of this biological system from the point of view of approaching it pharmacologically and there has been no agent demonstrated capable of accomplishing a clinical effect via this route.

We designed a novel type of molecular complex to achieve this. The agents used are various forms of a tripartite complex composed of a first moiety acting as an “axonal transport facilitator” (ATF), an “amplifying polymer” (AP) second moiety acting to achieve amplification of the fundamental event of synaptic endocytosis by carrying along

multiple drug molecules with each saturable uptake event, and a third moiety composed of multiple copies of the therapeutic molecule, reversibly linked to the polymer (Fig. 1).

We were able to administer a small intramuscular injection with $1/300^{th}$ of the usual oral dose of gabapentin (half life 8 hours) and achieve a degree of neuropathic pain suppression that could not be obtained with tolerable amounts of oral therapy while extending the half life of the drug by more than an order of magnitude.

MATERIALS AND METHODS

These are summarized in Table S-1 and presented in detail in the associated Supplementary Information that follows.

RESULTS

Results are summarized in table S-1

A. Chemical synthesis and stability

I – Chemical entities

[1]. Effective synthesis with loading sufficient for amplification

The synthesis strategy achieved attachment of drug to at least 30% of dextran monomers for both gabapentin and valacyclovir. The 70,000 MW dextran has approximately 430 dextrose sub-units so each Wheat germ agglutinin (WGA) molecule endocytosed in these experiments delivered around 100 molecules of drug.

[2]. Particle based carriers can be delivered intraneuronally

EDTA washing of dextran coated magnetite particulates resulted in hydroxide free ferrites that were non-reactive and preserved their superparamagnetic properties during axonal transport. After conjugation of an axonal transport facilitator to the dextran coat, they were effectively endocytosed and transported intact despite their (10-15 nm) size.

II – Intracellular release and drug activation

[3]. The stability of a drug is maintained through chemical linkage and biological release

In the BHK viral plaque reduction experiments, free drug in the culture medium resulted in plaque reduction by 96% at a dose of 5 micrograms/well, but a dose of 50 micrograms/well of the drug bound to dextran was required to achieve an equivalent effect. However we observed nearly 100% plaque reduction using a dose of 5 microgram/well of tripartite WGA-dextran-drug conjugate. This demonstrates that the

WGA efficiently promoted endocytosis of the tripartite agents while dextran-drug alone was not readily taken up by cells.

On chemical grounds, the carboxyl links binding drug molecules to dextran should be hydrolyzed after endocytosis. For valacyclovir (pharmacologically inactive), the product of the hydrolysis is free and active acyclovir. Therefore, the antiviral effect of the tripartite confirms release of acyclovir from the tripartite carrier after endocytosis.

B. Interactions with axon terminus and axonal processing

III. Effects of polymer, linker and drug

[4]. Effects of polymer size on tripartite uptake into nerves

10,000 MW dextran achieved more avid and rapid axonal uptake than 70,000 MW dextran. Dextran 70K based material is taken up effectively but at a lower rate. With 10K dextran, there is extensive uptake within ten minutes after application and washing. Similar levels of uptake are seen with 70K dextran after 60 minutes. However the differential efficacy of uptake was not large enough to outweigh the benefits of greater amplification of uptake events achieved by using the higher capacity larger molecules for drug delivery. Since molecular loading of the polymers was equivalent for various sized dextrans, the increased drug delivery with increased molecular size was linear. However,

the losses in uptake efficiency with larger molecules appeared to be much less than linear. The consequence is that although smaller dextran molecules were endocytosed more avidly and rapidly than 70K based vehicles, more drug molecules were still delivered by the 70K based vehicles.

[5]. Effects of charge on uptake and transport

The overall charge of the complex had a large impact on efficiency of uptake (Fig. S-5). There was virtually no uptake into cultured neurons when the overall charge of the complex was negative, and effective uptake occurred when charge was neutral, or when overall charge was positive.

[6]. Effects of side group mediated hydrophobicity on uptake and transport

Acylation of FITC-labeled, WGA-conjugated dextran to make the molecules progressively hydrophobic led to complete failure of uptake and transport. We assessed various degrees of acylation and found that this effect occurred even at very low ratios of acylation.

IV – Effects of axonal transport facilitator

[7],[8],[9]. Efficacy of physiologic, non-physiologic and synthetic ATFs

The Campenot chamber studies demonstrated similar efficacy for Nerve growth factor (NGF) and WGA for producing uptake of the tripartite carrying FITC (Fig. S-6). The phage display experiments demonstrated that novel purely synthetic ATFs could be discovered and then produced *in vitro* which not only had equivalent efficiency for neuronal uptake, but which could also be more efficiently sub-targeted to different types of neuronal populations (e.g. general sensory, pain, motor, autonomic). Blockage of movement of compounds with phage derived synthetic ATF by the use of axonal transport inhibitors confirmed that purely synthetic ATFs had been generated, identified, and produced.

V. Effects of intra-axonal processing

[10]. Survival of small chemical molecules linked to the tripartite

When WGA-dextran-gabapentin was used, cross staining with antibodies to gabapentin confirmed preserved antigenicity of the drug after axonal transport of the tripartite, although this method could not itself confirm that the drug was intact or active after transport and release. Dextran-gabapentin with no conjugated WGA did not produce detectable gabapentin antigenicity in the histological sections.

[11]. Large molecule access to spinal cord from intramuscular injections

After small IM injections in muscle, WGA delivered sufficient quantities of functioning HRP enzyme to label numerous motorneurons (Fig. 2a, 2b, 2c) in our primate model. Enzymatic activity of the horseradish peroxidase survived any hypothetical lysosomal degradation during transport and yielded product distributed throughout the neuron. Both epaxial and hypaxial muscles proved an effective route to reach spinal cord quickly (several hours) with no apparent difference in delivery of intact enzyme via axons despite the difference in distance of transport. The labeled spinal cord motor neuron pool for these axial muscles had greater longitudinal extent among spinal cord segments than for appendicular muscles. These findings are consistent with previous reports concerning WGA [20] and NGF [21] and their intramuscular introduction [22 23].

The rate of transport appeared to be consistent with time scales predicted by literature values of 30-100 mm/day [24]. Overall the area of spread of injectate was limited to a few millimeters, although the distribution in the epaxial muscle spinal cord motorneuron pool extended over several centimeters.

[12], [13], [14], [15]. Intact transport of targeted nanoparticles by intact axon termini

Electron microscopy showed appropriately sized ferrites within the axon, more than three centimeters from the WGA-dextran-Fe injection site (Fig. 4). The appearance

of the ferrites in endosomes was similar to what has been seen in studies of similar agents in other tissues [25], but in this case the particles were restricted to the axonal stream. The autoradiographic studies demonstrated sciatic nerve radioactivity and so provided similar evidence that the magnetite containing particles were transported in nerves.

The relaxivity experiments (Fig. S-4) taken together with distribution studies showing the concentration of magnetite delivered to the axon by the tripartite showed that the amount of magnetite transported should affect the T2 relaxation rate of nerve. The observation of a decrease of T2 relaxation time in nerves transporting superparamagnetic nanoparticles in both the micro-MRI nerve channel studies (Fig. S-7) and in the high resolution MRI experiments (Fig. S-8) confirmed that the carrier particles were not degraded. Any hydrolysis of the sub-domain sized particles would have eradicated their superparamagnetic effect on T2 relaxation time in nerve as transport progressed. The relaxivity effect far exceeded that which would result from free iron or ferritin at the doses administered.

The WGA-dextran-magnetite experiments in rabbits confirm other reports [26] that particles of 5-15nm are endocytosed and transported by intact nerve endings. Some studies have suggested that nerve injury is required for the transport of larger particles [27], but this result suggests that intact neurons will indeed transport large (up to 15nm) multi-molecular aggregates or “transport particles” after intramuscular injection when the particles are well solvated.

C. Targeting and pharmacological efficacy

VI. Clinical target access

[16], [17], [18], [19]. Targeted Access to Clinically Relevant Neuronal Sub-populations

Intramuscular injection of the tripartite WGA-dextran-FITC produced labeling of alpha motor neurons in the ventral horn and autonomic neurons in the intermediolateral cell column (Fig. 2d, 2e). We also observed good filling of proximal sensory neuron processes (axons) in the dorsal root entry zone and in lamina I and II of the dorsal horn of the spinal cord (Fig. S-9). Injection of foot pad and multiple hind limb muscle each resulted in labeling of less than 50% of ganglion cells, but injection of both muscle and skin resulted in filling of nearly 90% of dorsal root ganglion (DRG) cells (Fig. 2f, 2g, S-10). In clinical use, the objective will typically be to reach specific sub-populations rather than filling an entire ganglion *per se*, so these results support the expectation that a large fraction of cells in a e.g. a subpopulation innervating a single muscle or patch of skin can readily be reached.

Cross staining with an antibody to peripherin showed that many of the DRG sensory neurons that were accessed were C-fiber nociceptor cells (Fig. 3a to 3f). This helps confirm that this method of delivery does reach a selected subset of nociceptors that correlates specifically with the selected site of injection. The ATF played a major role since no detectable fluorescence was observed when dextran-FITC without ATF was administered in these experiments.

VII. Distinctive pattern of distribution relative to trans-vascular

[20]. Unique distribution with high fraction of drug reaching neuronal targets

In the whole body distribution studies with small calf muscle injections, concentrations of [^{125}I]-WGA in ipsilateral peripheral nerve and dorsal root ganglia of segments supplying the sciatic nerve reached six times systemic concentrations (Fig. S-11) and demonstrated saturability and transport time consistent with other reports [28]. Activity in spinal cord was less than in nerve or DRG but did reach twice systemic concentrations when higher concentrations of injectate were used. The greatest activities detected in other tissues were in liver and kidney and these apparently reflected metabolized iodine. There was no significant activity detected in contralateral nerve and DRGs nor in ipsilateral nerves, ganglia, or spinal cord related to cervical, thoracic, or higher lumbar segments. NGF showed less transport to spinal cord than was seen for WGA consistent with selective uptake of NGF by sensory and autonomic nerves. Detection of activity due to [^{59}Fe]-WGA-dextran- at other systemic sites was even less than for WGA or NGF alone.

Considerable amounts of drug remained in the axon itself at the time points sampled. It is known that para-nodal complexes of Schwann cells at the Nodes of Ranvier can endocytose materials from the axoplasm [29] and this may account for a peri-axonal depot effect with drug being cleared to the para-nodal complexes and then subsequently being re-released. Further studies will be needed to clarify this issue.

[21]. Site of injection retains high amount of injectate

The regional view provided by the [^{131}I]-WGA gamma camera studies demonstrates that the total amount of the injectate remaining at the site of injection is large when compared to the amount in the small volume of the nerve. In part, this reflects the fact that WGA adheres to muscle cell membranes [30] as well as being taken up by adsorptive endocytosis at nerve termini. This also explains a depot effect whereby additional amounts of the compound continue to be introduced into the axonal stream over a period of several days.

[22]. Pharmacologically efficacious drug amounts were delivered

The tripartite assemblage with [^{14}C]-labeled gabapentin produced activity levels for gabapentin in ipsilateral neurons of more than 600 times greater than background while counts remained at background levels in contralateral neurons. This demonstrated the localizing effect of injection of polymer-bound drug conjugated to an axonal transport facilitator, and also allowed us to estimate the drug concentration achieved based on the specific activity of the [^{14}C]-labeled gabapentin.

VIII – The pharmacologic effects of ATF mediated delivery are not obtainable by trans-vascular agents

[23]. Prolonged suppression of hyperalgesia

In the hyperalgesia experiments a single injection with the tripartite agent reduced the hyperalgesia ($p<0.05$ in eight out of the nine comparisons of treatment versus control) for at least four days after injection (Fig. 5). The total dose was 0.375 mg/kg

using the novel intraneural transport method compared to 120mg/kg total dose orally for four days of the usual human oral dosing. Prolonged efficacy may have been due to a depot effect in muscle, slow redistribution from axon and Schwann cells to nerve cell body, slow clearance from inside the nerve, or preemptive blocking of newly produced receptor molecules en route from ribosome to cell surface.

DISCUSSION

Gabapentin not only crosses the blood brain barrier but is subject to some active concentration [31,32]. Therefore, clinically efficacious axonal transport for intraneural drug delivery would need to produce similar concentrations near the internal cell surface of accessed neurons. In this model, histological estimates of the ratio between the estimated intracellular volume of the accessed neurons (InCvol) and the actual volume of the entire dorsal root ganglion (DRGvol) provide a correction factor (InCvol/DRGvol) that allows for conversion of total DRG concentration into estimated intracellular concentration in the accessed neurons.

When these considerations were employed, our counts of delivered [^{14}C] suggested delivery of gabapentin at intracellular doses equal to or greater than 100μM. These *in* vivo [^{14}C]-gabapentin studies therefore showed that this technology can deliver drugs to target neurons at pharmacologically efficacious doses using 1/300th of the oral dose that would be required to achieve the same intracellular concentration in target cells.

In addition, this system resulted in a ten fold increase in effective half life relative to oral administration. This appears to be because of the unique dynamics of this delivery mechanism. This involves both a muscle injection site depot effect for the large tripartite complexes and a prolonged stream of delivery to the neuron from the axon and its associated paranodal complexes.

In this set of experiments, neither systemic delivery of the agent by a subcutaneous route nor administration of free drug alone to the affected limb had any significant therapeutic effect. Although dextran can itself weakly promote axonal transport [33], the use of dextran/drug with no ATF showed only limited effect in occasional animals and was in no case statistically significant.

It had not been clear in the past whether gabapentin, delivered via axonal transport, acts inside nerve cells or on the external cell surface [34-36]. Further, the conjugation reaction and subsequent release of the gabapentin might have converted it to a chemically distinct metabolite or residue. The high efficacy of this intraneurally delivered agent constructed with gabapentin demonstrates for the first time that delivery of a clinically efficacious small molecule therapeutic agent from inside the cell via an intraneural route can achieve the same pharmacological effect as delivery from outside the cell via a systemic route. This also corroborates recent evidence that the site of action of gabapentin is indeed intracellular [37].

Whilst there are additional issues to resolve before axonal transport based medications come into regular clinical use their eventual development now seems realistic. Previously, the relatively small number of ‘uptake events’ per neuron for

molecules like NGF has limited their applicability for intraneural drug delivery. This work demonstrates that a linker carrying large numbers of drug molecules can amplify the pharmacological effect of each uptake event by at least two orders of magnitude. Amplification by an additional order of magnitude through alternative conjugation schemes appears to be readily achievable.

We anticipate that intraneural pain medication will have a significant impact on the management of pain after surgery and in the treatment of patients suffering from intractable chronic pain unresponsive to existing pain medications. Some efficacious agents whose use is limited by systemic toxicity may be safely and effectively delivered by an axonal transport delivery vehicle. It is likely that anti-viral, neuroprotectant and anti-spasmodic agents (small molecule, peptide, or nucleic acid) can also be delivered to important locations in the nervous system in this manner. The application of this new class of intraneural pharmacologic vehicles also provides a novel tool in the investigation of a number of aspects of basic neurobiology.

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Figures & Legends

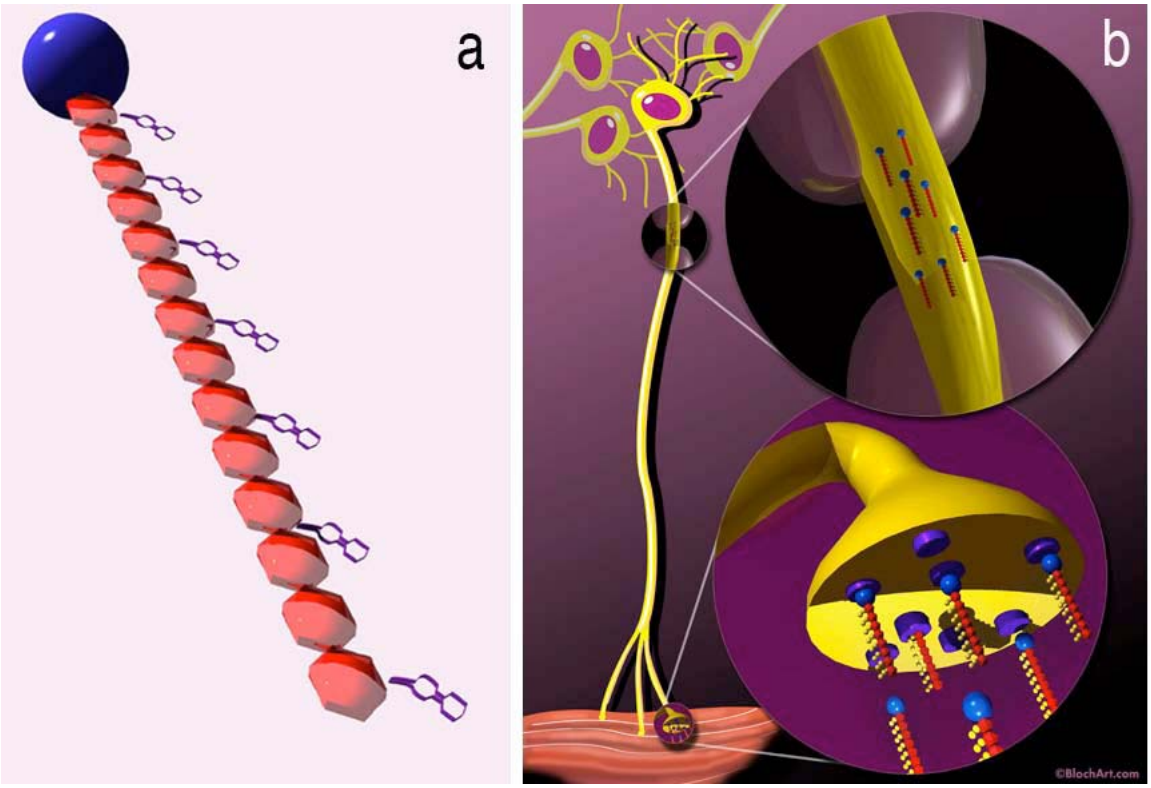

Figure 1 – Tripartite delivery vehicle.

(**a**) The drug delivery vehicles include a targeting element – the axonal transport facilitator or ATF (blue sphere), and a polymer such as dextran (red repeating units) that carry multiple drug molecules (purple). (**b**) They are designed to be injected in muscle or skin and then travel via an "intraneural" route to deliver the drug molecules to the cell body.

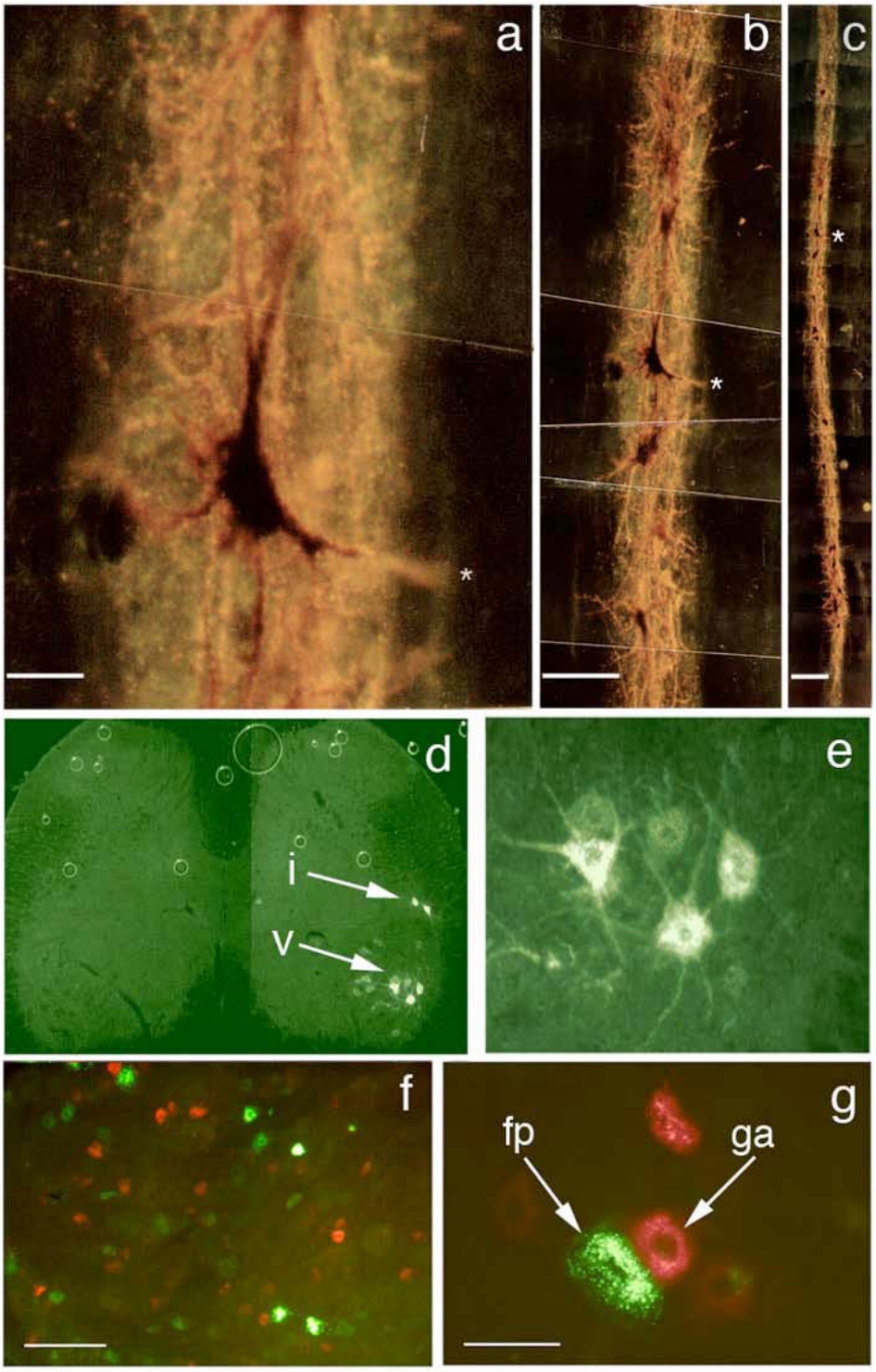

Figure 2 – Axonal transport to spinal cord neurons and dorsal root ganglion.

(**a-c**) Magnified, back illuminated view of an individual motor neuron in an oblique longitudinal section through a portion of the ventral horn of the spinal cord (*Macaca fascicularis*), and seen at lower magnification in figures b and c. The dark orange material seen inside the cell and filling the cell body and dendritic processes is the product of a chemical reaction carried out by an administered enzyme, horseradish peroxidase. To introduce this exogenous enzyme into the cell, it was conjugated to WGA, an ATF (axonal transport facilitator), then injected into a muscle innervated by the axons which arise from these neuron cell bodies. (**d**) Section of rat spinal cord showing retrogradely transported WGA-FITC in the motor neuron cell bodies (v) and in cells in the autonomic intermediolateral cell column (i). (**e**) magnified view of motor neurons seen in (**d**). (**f**, **g**) Delivery to rat dorsal root ganglion cells from different peripheral sources. Section of L4 dorsal root ganglia showing retrogradely transported FITC (green) injected intra-muscular and TRITC (red) injected intra-plantar, in the sensory neuron cell bodies (fp – footpad injection, ga – gastrocnemius injection). Scale bars (a) = 50 μm, (b) = 200μm, (c) = 400 μm (d) = 120μm, (e) = 30μm, (f) = 170μm, (g) = 45μm.

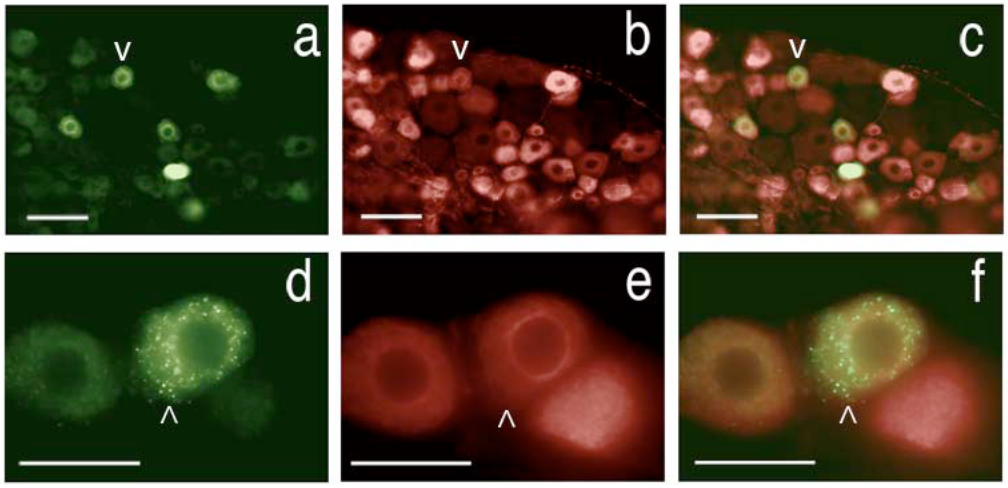

Figure 3 –Demonstration of transport to C-type nociceptor cells in dorsal root ganglion. Section of rat L5 dorsal root ganglia showing retrogradely transported FITC combined with immunohistochemistry for the specific C-fiber nociceptor marker, Peripherin. (**a**) Retrogradely transported FITC, (**b**) the same field as in a showing cells that are immuno–positive for peripherin, (**c**) overlay of c and d showing FITC is present in the nociceptors (v – same neuron in **a**, **b**, & **c**). (**d**) Two sensory neurons containing retrogradely transported FITC, a third neuron is unlabeled, (**e**) the same field as in c showing that all three neurons are positive for Peripherin, (**f**) overlay of f and g (^ - same neuron in **d**, **e**, & **f**). Scale bars (c-e) = 150μm, (f-h) = 40μm. 26 cells vs. 59 cells

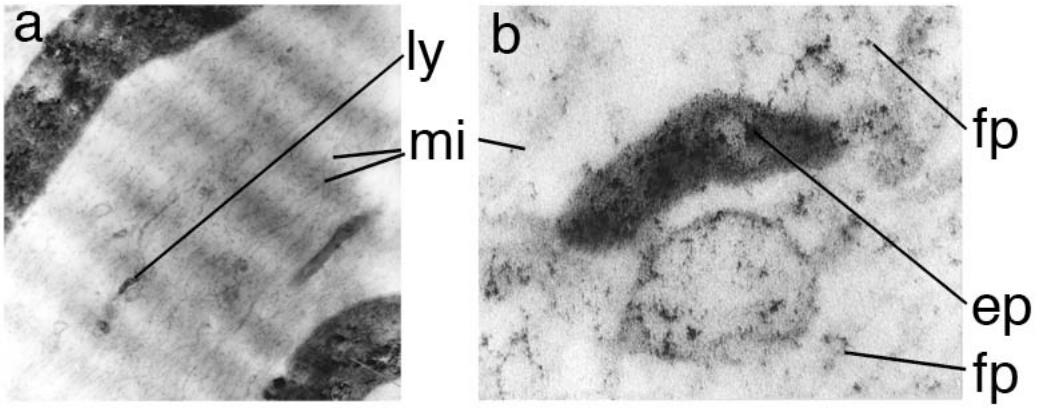

Figure 4 – Intra-axonal location of transported agents.

(**a**) Electron micrograph of rabbit tibial nerve four days after injection of the gastrocnemius muscle with ferrite-WGA tracer. (**b**) magnified view of a vesicle seen in (a) to 195,000x. (ly) lysosomal vesicle, (fp) small particles transporting on microtubules (mi), (ep) larger particles in vesicles.

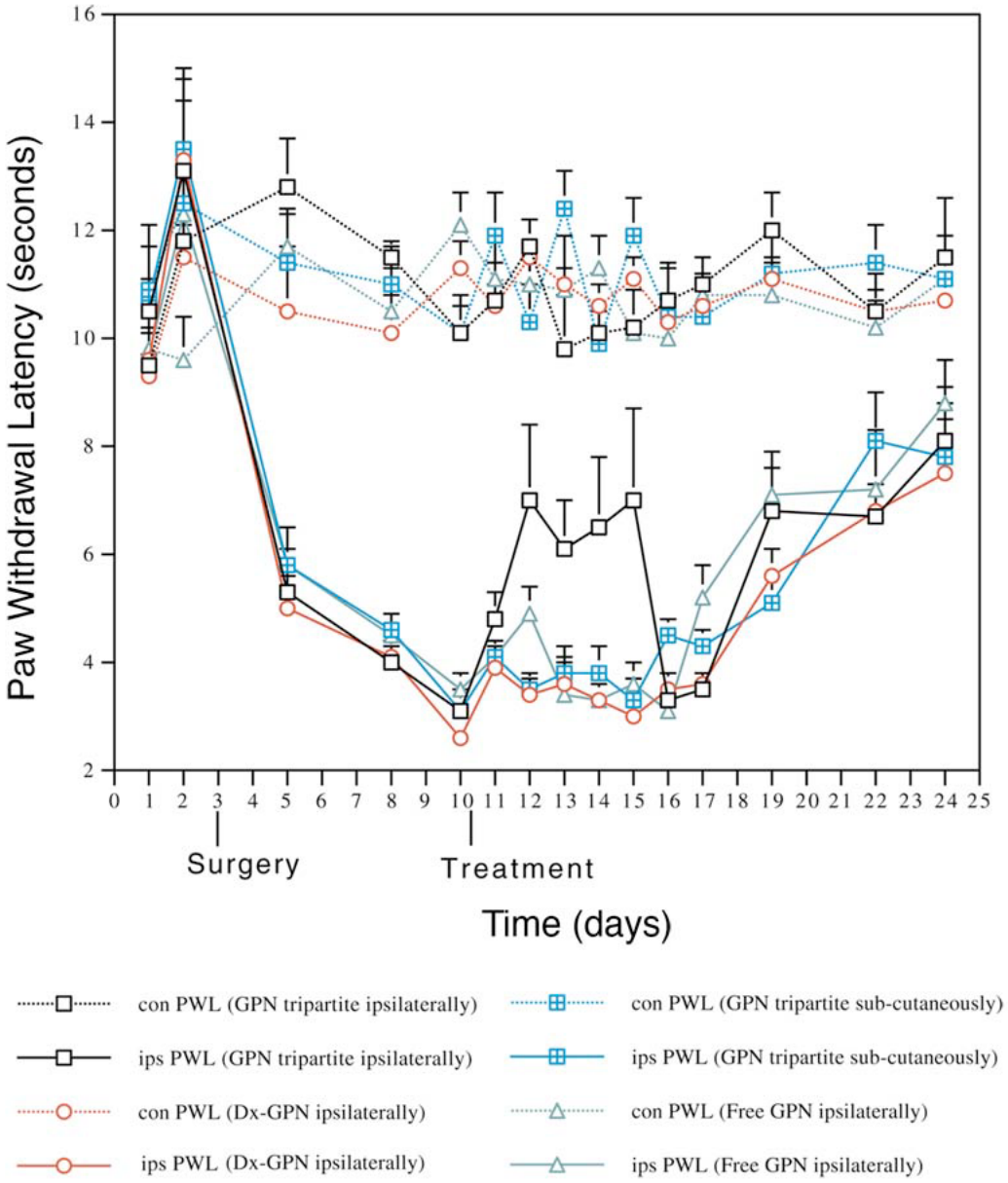

Figure 5 – Effect of gabapentin delivered by intraneural vehicle. The effect of Gabapentin tripartite administration, to mono-neuropathic animals, on thermal nociceptive threshold (con, contralateral; ips, ipsilateral; GPN, Gabapentin). Results are the mean ± SEM (n = 6). The difference between the ipsilateral

and contralateral side is significant from 4 days post surgery to the end of the experiment in all treatment groups. Treatment with Gabapentin tripartite to the injured limb caused a significant elevation in paw withdrawal latency which was evident within 2 days and lasted for a further 4 days, after which the latency returned to that of the other groups (e.g. day 12, P=0.0049 between Dx-GPN and GPN tripartite ipsilaterally). This effect was not observed in any other group.

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