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The Value of Antibodies as Research Tools in Neuroscience vs. their use as Distraction Agents

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ABSTRACT: In molecular and cellular neuroscience, antibody-based methods for target protein separation, characterisation, and localization are among the most important and frequently employed methods. The antibodies utilised in these experiments are frequently the results of arduous efforts by university and commercial neuroscientists to produce high-quality, well-characterized reagents for their own study. Our understanding of protein abundance, distribution, structure, and function has rapidly expanded as a result of several researchers making their antibodies available to other neuroscientists through generosity, cooperation, or commercial licencing. In an ideal world, antibodies would be created and verified through an iterative process that involves strict testing at independent labs and dissemination of pertinent information to the whole scientific community.

KEYWORDS: Neuroscience, Antibodies, Good Clinical Practice, Cellular Biology, Genome Project, Immunochemistry.

I. INTRODUCTION

One of the most significant and commonly used techniques in molecular and cellular neuroscience is the use of antibody-based techniques for target protein separation, characterization, and localization. The antibodies used in these studies are typically the product of laborious efforts by academic and industrial neuroscientists to generate high-quality, well-characterized reagents for their own research. Through charity, collaboration, or commercial licencing, some researchers have made their antibodies available to other neuroscientists, greatly advancing our understanding of protein abundance, distribution, structure, and function. Antibodies would be developed and validated in an ideal world through an iterative process that includes stringent testing at outside labs and sharing of essential information to the whole scientific community. Limitations of a specific antibody reagent would therefore be publicly acknowledged and would encourage efforts to produce additional antibodies with increased activity or specificity. Global accessibility to thoroughly described antibodies that may be dependably utilised to research a variety of characteristics of particular target proteins would be the end result. Such well characterised and verified antibodies are of immeasurable importance to the scientific community since they produce results that can be replicated in many laboratories, resulting in substantial advancements in the area. But the world is not ideal. In many cases, antibodies lack the necessary specificity and/or effectiveness. As a result, researchers may be forced to spend more money and time than necessary on study, and in the worst situation, they may start or continue false discoveries that cause widespread distraction. Proteomic techniques in neurology are receiving fresh attention as a result of the success of the Human Genome Project. In spite of their biochemical properties or natural abundance, a variety of target proteins may now be recognised by antibodies thanks to advancements in immunogen production, animal vaccination, and antibody formation and validation techniques. Additionally, a variety of commercial antibody companies have emerged and sell antibodies that are targeted at a variety of proteins, allowing individual scientists to concentrate on the use of antibody-based applications rather than the actual creation and validation process. Commercially produced antibodies serve as the main source of antibodies utilised in academic and industry research for particular protein classes. Commercial antibodies, unfortunately, frequently fail even the most basic tests of activity and/or specificity (such as selective staining of cells over-expressing the target protein versus those that do not express the target protein at all), and vendors frequently shift the responsibility for antibody validation and quality control to the end user. With the understanding that the vendor has

carried out sufficient quality control to demonstrate activity and specificity, the end user can choose to either use the antibody as is for her/his intended research goals or to become sidetracked from these objectives and invest time, money, and effort into conducting a critical evaluation of the antibody prior to use and eventual publication of their results. Researchers have expressed great displeasure with the subpar quality of antibody research tools in various domains of neuroscience, which has resulted in the dissemination and replication of false research findings. This is in response to the publication of results that were later found to be invalid due to the use of low-quality antibodies (Saper and Sawchenko, 2003). Journal editors must work to ensure publication of results that will withstand scrutiny in order to prevent the spread of incorrect findings. Even though the JCN's requirement to use limited research resources (including stocks of antibodies) in assays aimed at simply validating the specificity of commercially obtained, expensive research resources may seem extreme and draconian to some, it is necessary. This is a noble objective, but it can be beyond the means of individual researchers who lack expertise in antibody creation and characterization or who have staffing or budget restrictions that prevent them from pursuing costly tools and their extensive validation.

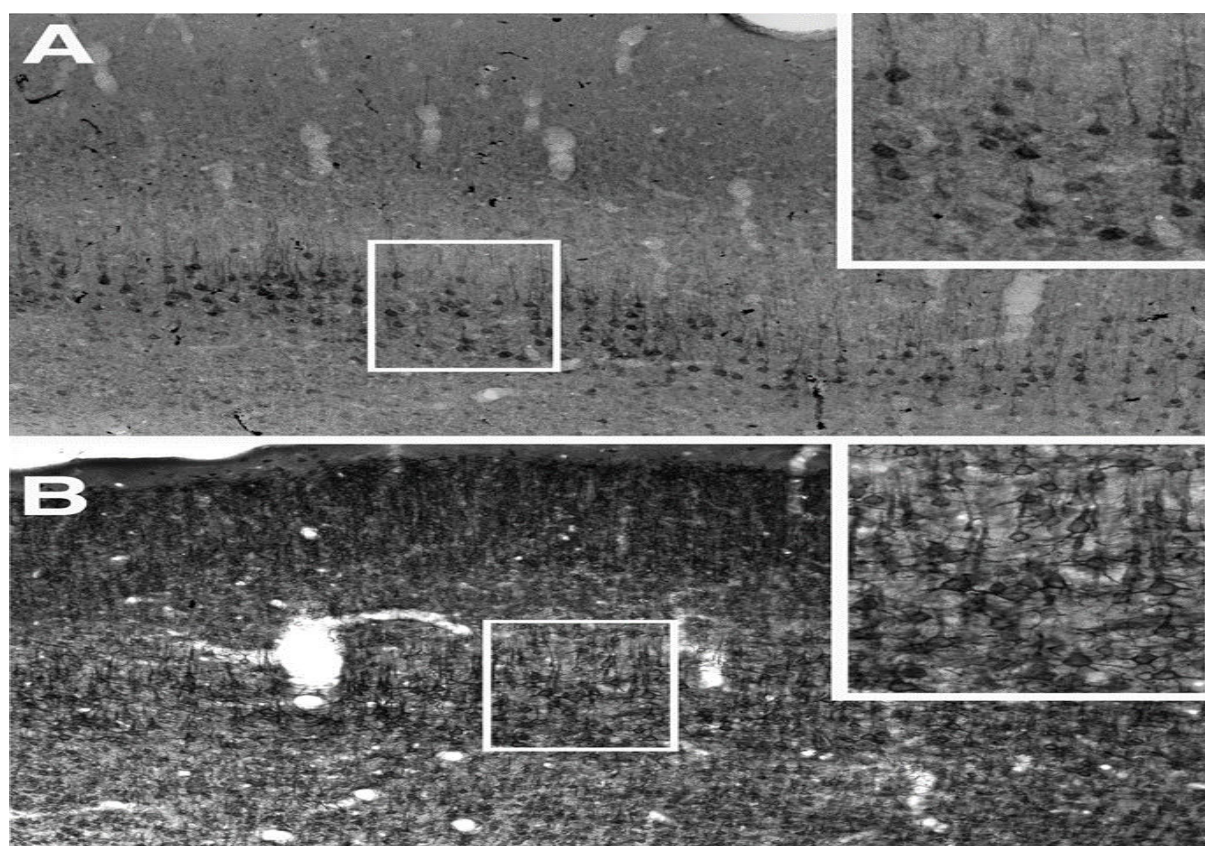


Figure 1 illustrates examples of nonspecific and selective somatodendritic ion channel nickel-enhanced diaminobenzidine staining from adult rat neocortex. **A**, A typical non-distinctive staining pattern. In one instance, a Kv3.1-specific mAb that was ELISA-positive stained the cytoplasm rather than the plasma membrane in neocortical layer V pyramidal neurons, which do not express Kv3.1 mRNA (Weiser et al., 1994). **B**, Kv2.1-specific staining. Take note of the distinct ion channel-associated staining at the plasma membrane and the lack of cytoplasmic or nuclear signal. Views of the boxed region at greater magnification are shown in insets.

Therefore, the issues that need to be addressed are as follows: how can an investigator who may have little to no experience in antibody characterisation utilise an antibody to address a particular research topic and proceed with confidence in the outcomes? How much antibody characterisation should an investigator do in their own lab before submitting their findings for publication? What is the best place to offer constructive criticism to commercial antibody businesses (and academic laboratories) in order to persuade them to describe and validate their products for research usage in more detail? Affinity reagents are antibodies. Although high-quality antibodies will have a high affinity (low nanomolar KD corresponding to 0.1-1 g/ml for IgG), and consequently a high degree of efficacy and specificity for their target antigen, even the best antibodies can exhibit cross-reactivity with "off-target" antigens at high antibody

concentrations or in complex protein mixtures (such as brain membrane extracts, cell lysates, or brain sections). Even though even the most highly purified polyclonal antibody (pAb) preparations (i.e., affinity-purified antibody preparations) may contain anti-bodies with reactivity against closely related antigenic sequences, in some cases this crossreactivity contributes to or even dominates the observed signal. This is true even when using crude polyclonal antisera to produce high-quality and reliable results. Contrary to polyclonal antisera, in which the pAb of interest is a minor component of the total serum antibody pool, the techniques used to produce and purify monoclonal anti-bodies (mAb) have the advantage that the mAb is either the sole or vastly pre-dominant antibody present in the material with which to begin purification. One must carefully choose the criteria and settings under which the specificity of the antibody reagent is most likely to be established in order to avoid off-target binding when using the many types of antibodies in cellular and molecular neuroscience.

II. IMMUNOHISTOCHEMISTRY

When antibodies are employed for immunohistochemistry (IHC), especially on slices of brain tissue, their limitations become most obvious. This is primarily due to the complex and heterogeneous cellular and molecular makeup of the brain in comparison to other tissues, as well as the fact that IHC lacks the cues used in other experimental settings to assess the specificity of an antibody signature, such as reactivity against a single entity of the proper molecular mass or electrophoretic mobility. It is difficult to determine whether staining is specific (i.e., indicates the presence of the target antigen) and which is nonspecific since reaction product or fluorescence displays all places at which the antibodies have attached (closely related or unrelated antigens). Therefore, To guarantee that the interpretation of the antibody staining pattern is accurate, one must search for convergence and concordance of data collected using a variety of approaches when applying antibodies in IHC. Applying a mix of in situ hybridization (ISH), histochemistry, and IHC procedures is one way to get convergent data. One can determine the brain areas and cell types that are expressing mRNA encoding the target antigen by carefully using ISH histochemistry or carefully analysing ISH data, either in publications or in online atlases (for example, the Allen Brain Atlas; www.brainatlas.org). A context for interpreting the IHC staining pattern is provided by the mRNA distribution; if the IHC signal is localised to somata or dendrites, the ISH signal should be present in the same brain regions and cell types. An investigator familiar with the neuroanatomical connections should be able to assess whether the staining is in routes or terminal fields that come from cells expressing the relevant mRNA if the IHC signal is confined to axonal processes. Such congruent information from two independent approaches offers a very strong affirmation of the antibody signal (Rhodes et al., 1996). The target protein's purpose and logical subcellular location inside the neuron must also be kept in mind. In contrast to transcription factors, which should show nuclear staining, plasma membrane channels and receptors should produce strongly defined plasma membrane and not diffuse cytoplasmic staining (Fig. Comparing the IHC staining patterns produced by two separate antibodies prepared to recognise unique, nonoverlapping epitopes on the same target protein is another method for generating consistent results. Areas of tight overlap in IHC staining for the two antibodies are extremely likely to provide a specific signal since it is exceedingly improbable that antibodies produced against nonoverlapping antigenic sequences from the same target antigen will display the same specific and nonspecific staining patterns.

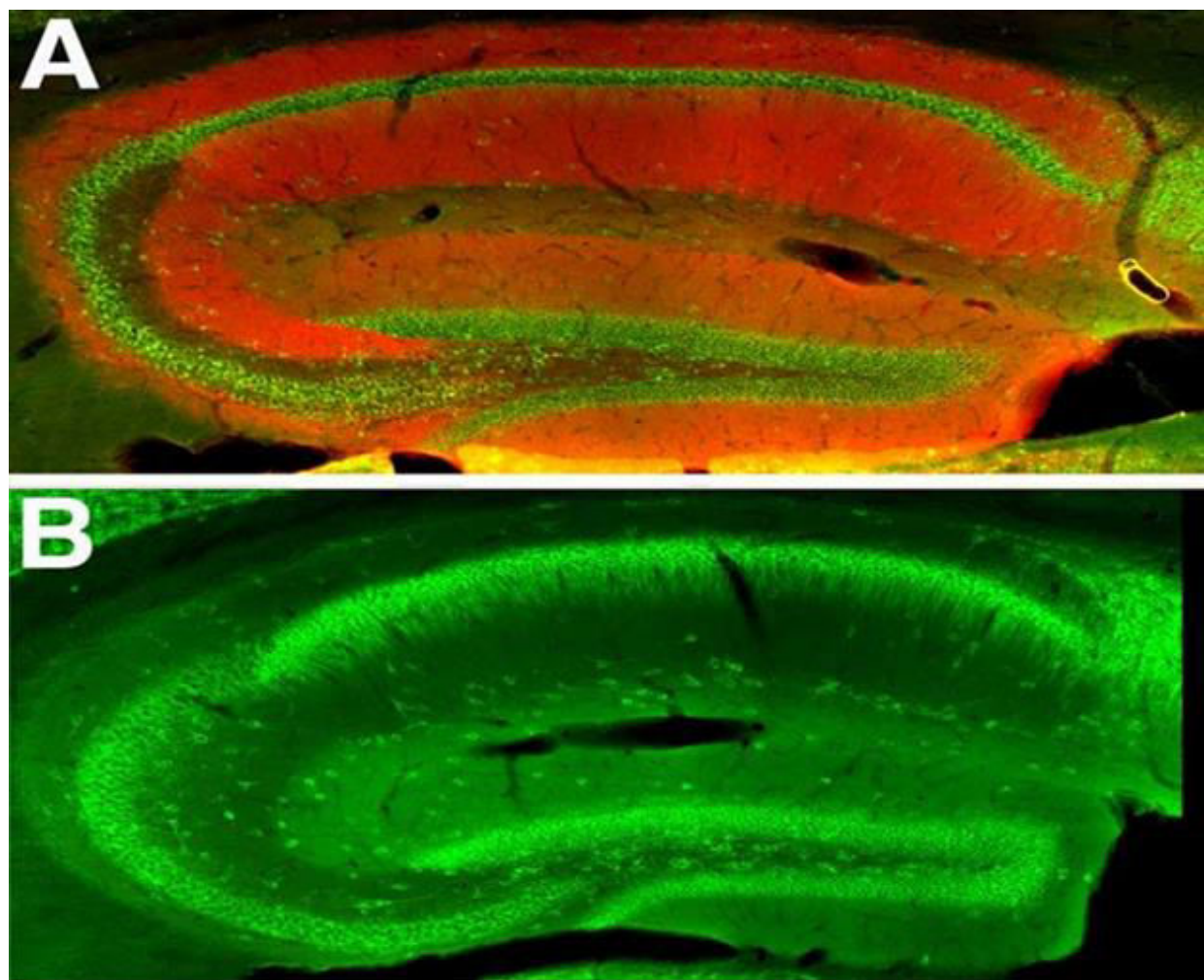


Figure 2: Using knock-out mice, antibody labelling for a dendritic ion channel was validated. Hippocampi from adult wild-type (A) and Kv4.2 knockout (B) mice were stained with an anti-Kv4.2 mAb (K57/1; red) and an anti-Kv2.1 pAb (green) in a specific pattern.

Because it is very simple to create several cRNA or cDNA probes targeted at non-overlapping regions of the target mRNA, this method of mRNA localization confirmation is frequently utilised for ISH. Researchers have created high-quality anti-bodies to separate sections of the target protein, despite the fact that it can be significantly more difficult and is only sometimes done. One of the most effective methods for verifying the IHC signal's specificity is this one. Multiple, independent mAb clones with unique, nonoverlapping binding sites on the same target protein are frequently produced by mAb projects; convergence of staining patterns acquired with such a panel of mAbs can produce a strong confirmation of specificity. As a broad approach for antibody validation, monoclonal antibody screening Getting high-quality antibodies for IHC might be very easy or exasperatingly difficult. There are multiple instances of mAb projects where almost every mAb acquired produces stunning IHC, as well as crude polyclonal antiserum that has sufficient specificity to be utilised for IHC without further purification. To locate even one mAb or pAb that yields adequate IHC staining, it frequently takes laborious, multistep purification/adsorption stages (for pAbs) or studies of a large number of otherwise positive clones (for mAbs). We have tested more than 5000 ELISA-positive mAbs throughout the years (in more than 100 mAb studies) using IHC labelling on slices of mammalian brain. Initial screenings often demand for a high-throughput format (like ELISA) that can handle a lot of samples (about 1000 for a typical mAb research). We specifically screen 50 to 100 ELISA-positive clones for IHC staining of mammalian brain slices in addition to other screenings (immunofluorescence staining of transfected cells, immunoblotting of brain preparations) (Bekele- Arcuri et

al., 1996). Only a tiny fraction (1–5) of the 50–100 clones that tested positive by ELISA were able to produce excellent results when IHC stained brain slices, and these clones often did not match to the strongest ELISA-positive clones. In light of this, we advise that researchers request and test as many ELISA-positive clones as is practicable for those that contract out mAb manufacturing in order to get a reagent that can be utilised for IHC. Standard controls should be used, as beautifully outlined by Saper and Sawchenko (2003). These controls include the absence of primary and secondary antibodies, immunogen competition, etc. Such tests should be carried out throughout the whole mAb assessment process (subcloning, mass manufacturing, and purification), and the same is true for pAb preparations (crude sera, IgG fractions, affinity-purified preparations, etc.). In order to further confirm the specificity of the observed staining patterns, additional evaluation by comparison with mRNA distribution, staining in sections from knock-out animals in which the target antigen has been genetically deleted (Fig. 2), or expression levels that have been attenuated by siRNA, can and should be used, when available. The greatest proof of anti-body specificity for all antibody reagents comes from the absence of IHC signal in tissues from knock-out antibodies when the antigen has been eliminated. At the University of California Davis/National Institute of Neurological Disorders and Stroke/National Institute of Mental Health NeuroMab facility, which is supported by the National Institutes of Health, we recently put the above-described methodology into practise to systematically generate mAbs for significant neuroscientific targets. With an emphasis on mammalian-brain immunoblotting and IHC, it is intended to produce high-quality mAbs that are validated using the aforementioned techniques. The confirmed NeuroMabs will then be made freely available to the scientific community. Although this one facility, whose aim is to produce NeuroMabs against 50 targets annually, cannot immediately meet the urgent antibody requirements of the field of neuroscience, it may serve as a model for concurrent academic or industrial efforts aimed at producing high-quality antibodies for neuroscience research through meticulous, systematic validation procedures, including rigorous brain IHC, allowing end users to concentrate on experiments requiring their respective neoantigens. Instead of becoming sidetracked by putting in crucial time and effort on antibody validation/quality control, which should be done before to distribution rather than afterwards. The NeuroMab website (www.neuromab.org) has started a message board for comments on end users' experiences using NeuroMabs and other widely available antibodies from nonprofit or commercial sources for neuroscience applications in order to better disseminate information about the dependability of antibodies for neuroscience research. This should enable researchers to find antibodies with a solid track record in the neuroscience community and prevent them from wasting time and resources on antibodies that have a track record of producing subpar outcomes.

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