

A harmonized segmentation protocol for hippocampal and parahippocampal subregions: why do we need one and what are the key goals?

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/hipo.22671

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Running title: A harmonized hippocampal subfield protocol: key goals and impact

Number of text pages: 9

Number of tables: 0

Number of figures: 2

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Funding sources

NR is supported in part by NIH grant R37 011230, ADE NIH/NINDS grant NS076856 and NS093052 and the UC Davis Chancellor's Award, TAS a post-graduate fellowship award from Eisai and the Canadian League Against Epilepsy (CLAE), AB by NIDA K01 DA034728, KMK by NIH/NIA R00 AG-036818, LW by Alzheimer Association grant ADNI 2-12-233036, RI by Grant GI20152980, MAY by US NIA P50 AG05146, NIA R21 AG049220, and NIMH R01 MH102392, MMC by National Sciences and Engineering Research Council Canada, Canadian Institutes of Health Research, Weston Brian Institute, Alzheimer's Association, Michael J. Fox Foundation for Parkinson's Research, and Fonds de recherche santé Québec and KMR by NIA R00 AG-36848, and PAY by NIA R01 AG037376 and NIBIB R01 EB017255, and RLJ by Fondation Thérèse et René Planiol pour l'étude du Cerveau

Keywords: Hippocampus, segmentation, harmonization, MRI, parahippocampal gyrus

Disclosures

None of the authors has any disclosures, except GAK, who is an employee of Genentech, Inc.

Abstract

The advent of high-resolution magnetic resonance imaging (MRI) has enabled *in vivo* research in a variety of populations and diseases on the structure and function of hippocampal subfields and subdivisions of the parahippocampal gyrus. Due to the many extant and highly discrepant segmentation protocols, comparing results across studies is difficult. To overcome this barrier, the Hippocampal Subfields Group was formed as an international collaboration with the aim of developing a harmonized protocol for manual segmentation of hippocampal and parahippocampal subregions on high-resolution MRI. In this commentary we discuss the goals for this protocol and the associated key challenges involved in its development. These include differences among existing anatomical reference materials, striking the right balance between reliability of measurements and anatomical validity, and the development of a versatile protocol that can be adopted for the study of populations varying in age and health. The commentary outlines these key challenges, as well as the proposed solution of each, with concrete examples from our working plan. Finally, with two examples, we illustrate how the harmonized protocol, once completed, is expected to impact the field by producing measurements that are quantitatively comparable across labs and by facilitating the synthesis of findings across different studies.

Introduction

The medial temporal lobe (MTL) in general, and the hippocampus in particular, have attracted extensive interest in *in vivo* human studies of a wide range of diseases (e.g. (Small et al., 2011)), normal life-span development (e.g. (Daugherty et al., 2016)) and cognitive abilities (e.g. (Squire et al., 2004;Van Petten, 2004;Carr et al., 2010;Kyle et al., 2015a)) involving structural (e.g. (Mueller et al., 2008)) as well as functional magnetic resonance imaging (MRI) (e.g. (Yassa et al., 2010)). The MTL includes the hippocampal formation and several cortical regions within the parahippocampal gyrus, namely the entorhinal, perirhinal, and parahippocampal cortices. The hippocampal formation itself is a complex structure that is composed of distinct subfields—the subicular complex (including prosubiculum, subiculum proper, presubiculum and parasubiculum), Cornu ammonis (CA1-CA4), and the dentate gyrus (DG)¹ (e.g. (Duvernoy et al., 2005;Insausti and Amaral, 2012)). In the early 2000's, *in vivo* measurement of hippocampal subfields first became possible through advancements in neuroimaging and analysis techniques (Small et al., 1999;Small et al., 2000;Zeineh et al., 2000;Zeineh et al., 2001). Since then, over 20 manual protocols (e.g. (Insausti et al., 1998;Small et al., 1999;Zeineh et al., 2001;Kirwan et al., 2007;Mueller et al., 2007;Ekstrom et al., 2009;Olsen et al., 2009;Kerchner et al., 2010;La Joie et al., 2010;Malykhin et al., 2010;Preston et al., 2010;Yassa et al., 2010;Bonnici et al., 2012;Libby et al., 2012;Wisse et al., 2012;Bender et al., 2013;Palombo et al., 2013;Winterburn et al., 2013;Suthana et al., 2015); see also (Yushkevich et al., 2015a)) and several automatic procedures (e.g. (Fischl et al., 2009;Van Leemput et al., 2009;Yushkevich et al., 2010;Augustinack et al., 2013;Pipitone et al., 2014;Iglesias et al., 2015;Yushkevich et al., 2015b)) have been developed for *in vivo* segmentation on MR images of hippocampal and parahippocampal subregions. These manual and automatic segmentation protocols are highly discrepant from one another and often employ different terminology and definitions of the

¹ Note that the terminology for MTL structures differs per neuroanatomy laboratory. For example, some neuroanatomists prefer the term CA4 (Duvernoy et al., 2005), whereas others refer to this region as hilus (West and Gundersen, 1990) or part of CA3 (Insausti and Amaral, 2012).

regional boundaries (Yushkevich et al., 2015a). This variability in segmentation protocols produces widely inconsistent results, even when studying similar populations and phenomena (e.g. (de Flores et al., 2015a;Wisse et al., 2015)). This variability complicates meaningful comparison of results between studies (de Flores et al., 2015a) and hampers the ability to draw broader theoretical conclusions. Thus, there is a strong need for a harmonized protocol that can be employed consistently across laboratories. Due to the wide variability in segmentation protocols and the lack of consensus in the field, no single protocol can be readily adopted as a common standard. Instead, it is necessary to develop a new manual segmentation protocol via consensus from the scientific community that resolves the various discrepancies and consolidates the commonalities across procedures and neuroanatomical reference sources. Although automated methods are appealing for efficiency and ease of adoption across research groups, the current automated protocols also differ widely from one another (Yushkevich et al., 2015a) and, when pitted against the “gold standard” of manual segmentation protocols, show limited concurrent validity, especially for smaller subfields. Automation of subfield segmentation therefore remains a hopeful future goal, but the first necessary step in its development is to establish a harmonized protocol for manual segmentation.

Inspired by the European Alzheimer’s Disease Consortium (EADC) - Alzheimer’s Disease Neuroimaging Initiative (ADNI) Harmonized Protocol (HarP) project (Boccardi et al., 2015;Frisoni et al., 2015), which successfully developed a reliable harmonized segmentation protocol for labeling the whole hippocampal formation as a single structure on 1 mm³ T1-weighted images, the Hippocampal Subfields Group (HSG) was formed in 2013 with the aim of developing a harmonized protocol for the segmentation of hippocampal subfields and the adjacent entorhinal, perirhinal and parahippocampal cortices (Kivisaari et al., 2013) for high-resolution T2-weighted 3 and 7 tesla (T) MRI data. T2-weighted imaging is the most commonly used for subfield segmentation because the stratum moleculare laconosum radiatum that is visualized as a “dark band” is used to separate the CA and the DG; this band

is less visible on T1-weighted images. As one of the first steps towards harmonization, 21 segmentation protocols for hippocampal and parahippocampal subregions were compared directly and revealed a range of similarities and differences between protocols (Yushkevich et al., 2015a). The greatest disagreement between protocols in the hippocampal body, for example, was in the demarcation of the CA1-subicular complex boundary (see Figure 1). This comparison underscores the urgent need for a harmonized protocol.

Since publication of this comparison paper in 2015, the HSG has developed a working plan for creating a harmonized protocol for high-resolution T2-weighted MRIs. In short, it includes: 1) localizing subfield boundaries on histology in a reference set of multiple *ex vivo* specimens, 2) deriving specific rules for placing boundaries on *in vivo* MRI using this histological reference material, 3) sharing the protocol with the larger community to solicit feedback, and 4) performing a formal reliability analysis on the agreed manual segmentation protocol. These steps are illustrated in Figure 2. This working plan reflects three key goals: attaining content (anatomical) validity, establishing measurement reliability, and enabling application to study normative and pathological changes across the lifespan. These key goals are discussed below in the context of the unique challenges posed in this venture and the proposed working plan for achieving each goal. In addition, we elaborate on the expected impact of this harmonized protocol on the field.

Key goals and challenges in the development of a harmonized segmentation protocol for hippocampal and parahippocampal subregions

Content Validity: Agreement with Anatomy

The harmonized protocol must achieve content validity for anatomy confirmed by various histological reference materials. Although extant segmentation protocols adhere to printed neuroanatomical atlases (e.g. (Duvernoy et al., 2005; Mai et al., 2008; Insausti and Amaral, 2012)), they largely rely on different

reference materials (Yushkevich et al., 2015a), which may have, in part, contributed to the variability amongst protocols. In addition to simply providing different images and different anatomical definitions, most references depict a limited number of slices along the anterior-posterior length of the hippocampus. Furthermore, the angulation of histology samples commonly differs from the orientation of MRI, thereby limiting their usefulness as the anchors of validation. Moreover, printed anatomical atlases often show only a limited number of specimens, making approximation of individual differences in anatomical landmarks and variability of subfield boundaries along the anterior-posterior axis of the hippocampus difficult to assess.

Approach: To resolve the outlined challenges, at least three neuroanatomy laboratories (e.g. KA, RI & JCA) will provide a specimen, processed according to the standards in the specific labs, and all three labs will annotate each sample (Step 1)—thus creating a variety of samples whilst allowing direct comparison of anatomical boundary demarcation between neuroanatomists. Notably, subfield boundary placement may differ between neuroanatomists, potentially arising from differences in processing methods or their own subfield definitions. While resolving these potential discrepancies is beyond the scope of our effort, we will use all available information and characterize the range in which the boundaries may fall to develop a protocol for MRI. Moreover, the use of different processing methods will broaden the applicability of the harmonized protocol. Critically, the samples will include multiple slices spanning the anterior-posterior length and sectioning will be oriented similar to common neuroimaging protocols (i.e. perpendicular to the long axis of the hippocampus (Mueller et al., 2007; Yushkevich et al., 2015a)). This reference set will be the most comprehensive dataset to date and will be a good starting point, used in addition to canonical atlases, to develop a protocol for *in vivo* MRI segmentation. Although unique and comprehensive, this atlas set only includes 3 samples because of practical constraints—e.g., the labor intensive nature of annotating histological samples, the available time of the expert neuroanatomists and the availability of data either still intact or cut perpendicular to

the long axis of the hippocampus, which is not a common neuroanatomical procedure. Therefore, existing histological references materials will also be used throughout the process and the neuroanatomists and MRI groups will continue to consult throughout the protocol development, particularly as it relates to individual differences in anatomical features.

Optimizing measurement reliability while maintaining anatomical validity

As important as the content validity vis-à-vis the anatomy is, the harmonized protocol must also produce reliable volumetric measures. High inter-rater reliability is particularly essential for this protocol as it is meant to be adopted and applied uniformly across groups. Because many of the features used to determine hippocampal subfield boundaries *ex vivo* (e.g., cell shape, size or density) cannot be visualized on high-resolution MRI, attempts to directly replicate anatomical boundaries commonly result in unreliable measures. An example is the endfolial pathway (Lim et al., 1997) in the stratum oriens of CA3, composed of loosely packed cells, which forms the inferior border of the CA3 curving into the DG. Although this border has been reported to be discernable on 7T MRI (Parekh et al., 2015), this border cannot be consistently observed on the typically-used lower-resolution 3T scans and might therefore lead to unreliable measurements. The use of a geometrical rule for this border on 3T data may therefore be necessary. Given the limitations of MRI resolution and quality, some small compromises in neuroanatomical precision are to be expected as boundaries must be reliably reproduced. Thus, the HSG endeavors to create a protocol that strives to maximize the approximation of the MTL anatomy while affording high reliability of its measurement.

Approach: Toward this end, we will develop a manual segmentation protocol for T2-weighted MRI based on the comprehensive anatomical reference set, while testing reliability at multiple stages. In Step 2, we will derive heuristic rules from the anatomical reference set and, via comparison to MRI, determine landmarks available in the vast majority of *in vivo* T2-weighted MR images that are commonly

used for hippocampal subfield segmentation. The draft protocol will be tested for reliability and may be subsequently re-tested until sufficient reliability is attained (Fig 2), based upon criteria reported in the literature and if needed, by majority voting. Additionally, following critical feedback from the larger community (Step 3), we will perform a formal reliability analysis of the final protocol in Step 4 with six raters drawn from across laboratories which will ensure that the protocol can be adopted by different research groups.

Broad application of the harmonized protocol

A final key goal is to develop a harmonized protocol that can be adopted widely for the study of populations across the lifespan and across several diseases. There are several practical challenges to achieving this goal: differences in scanning platform and acquisition protocols, variable scan quality (e.g., common motion artifacts in MRI scans of children or patients with Alzheimer's disease), segmentation goals of a particular study (e.g. the number of hippocampal subfields or cortical regions of interest) and potential differences in the hippocampal subfield boundary locations due to alterations in the internal composition of the hippocampus in certain populations.

Approach: We have incorporated a number of solutions in our working plan to ensure that the harmonized protocol meets the requirements and needs of different research groups. First, to allow for the highest level of adoption across research groups, we aim for the harmonized protocol to include definitions to separately segment the following subfields: subicular complex, CA1, CA2, CA3, and DG (including Fascia dentata and CA4), and the parahippocampal, perirhinal and entorhinal cortices. Such delineation will be contingent upon the achievement of high reliability and validity for each region. The protocol is intended to remain flexible for the user to decide which subfields to potentially collapse into a single measurement (e.g., the common inclusion of CA3 with the DG) depending on the particular research goals. Of note, we deliberately limit our protocol to the selected subfields in an effort to

maintain high reliability of all measures, as the amount of detail on *in vivo* MR images is insufficient, in our opinion, to segment additional structures at 3T. Developing a protocol for 3T data is the first focus of the group as it is most common in the field. Upon completion of the 3T harmonized protocol, it will be adjusted for other applications, such as 7T structural images, which may involve adding more fine-grained demarcations to the 3T protocol.

Second, we will develop and test our protocol in two different datasets consisting of scans from different research groups and reflecting the different ages and diseases most commonly studied in relation to these MTL regions. More specifically, it will include the age range from 7-100 years, and scans from healthy subjects as well as patients with hypertension, epilepsy and Alzheimer's disease. Importantly, these images also reflect the different types of 3T scanner manufacturers and sequences used in the field, as well as commonly observed imaging artifacts. Relatedly, by using anatomical features that are observable on these MRI scans and rules that can fit hippocampi with varying shapes, the protocol will be able to accommodate variability in hippocampal morphometry that occurs in development and diseases such as epilepsy and Alzheimer's disease, as has been shown to be feasible by previous segmentation work in these populations (Mueller et al., 2010; Daugherty et al., 2016; Santyr et al., 2016). However, our MRI dataset does not include all possible MTL abnormalities that can occur due to injury or disease (e.g. encephalitis or prenatal/postnatal abnormalities that may affect the folding of the hippocampus (Rosenbaum et al., 2014)). We will therefore additionally leverage the collective experience of the large research community contributing to this effort, including members who have first-hand experience with segmentation in special cases of MTL abnormalities. Additionally, it should be noted that we currently only have access to histological material from older adults, but not to histological material from younger age groups or some of the diseases of interest in our effort. This may limit our ability to confirm anatomical validity of our protocol when applied to various pathological conditions that may plausibly alter the internal composition and shape of the hippocampus. When

evaluating the validity of the protocol, we will therefore complement our histological dataset of healthy brains with histological reference material from pathology (e.g. (Insausti et al., 2010) whenever feasible. In the application to the study of development, we have a reasonable expectation that a similar protocol can be applied to samples of children and adults. Although hippocampal structure and morphometry continue to develop into adolescence (Insausti et al., 2010), the cytoarchitectonic differences that define the subfields as observed in the adult brain can be seen as early as mid-gestation (Humphrey, 1967; Arnold and Trojanowski, 1996) and are almost adult-like from the first postnatal year on (Insausti et al., 2010). Additionally, some cells in the hippocampal formation take on adult-like qualities between the 2nd and the 8th year (Seress et al., 2001; Seress, 2007). However, in the future, if more histological datasets become available with sectioning perpendicular to the long axis of the hippocampus, this may allow the validation of this protocol in younger age groups or certain disease populations and potentially necessitate updating the protocol.

Third, the HSG is a large, international collaborative group (approx. 150 members from more than 15 countries) that represents all levels of expertise and experience with different types of data, and different research interests. The HSG community will be asked for input in two stages. In Step 2, the boundary working group members (approx. 30) that are not involved in developing the *in vivo* definitions will be asked for initial feedback. In Step 3 feedback will be solicited from the larger community via an on-line questionnaire, similar to the Delphi procedure used in the HarP project (Boccardi et al., 2015). To further increase the versatility and expertise of the HSG, the authors invite other researchers to join this open effort and to provide input (www.hippocampalsubfields.com/mailling-list/).

Impact of a harmonized segmentation protocol for hippocampal and parahippocampal subregions

We expect that this harmonized protocol will have a significant impact in the research community as its use will enable direct comparison of results between studies, thereby better accommodating the replication of results and the pooling of data for meta-analyses. Existing data could potentially be re-analyzed with the harmonized protocol and we encourage researchers to consider this approach as an avenue for reconciling current discrepancies in the literature. Additionally, capitalizing on a uniform segmentation protocol adopted across research groups, the field can gain further insight into lifespan developmental trajectories and related diseases on hippocampal and parahippocampal structure and function. We provide two compelling examples of this in the study of aging and of pattern separation and completion for which the harmonized protocol could specifically be of added value.

Example 1: Age-related hippocampal subfield atrophy

Characterizing effects of advanced age on hippocampal subfield volumes is of great importance for understanding typical cognitive decline and departure from normal trajectories in the course of disease. Unfortunately, so far results pertaining to the association of age with hippocampal subfield volumes are inconclusive, with studies finding an effect of ageing on virtually all possible combinations of subfields (e.g. (Mueller and Weiner, 2009;Shing et al., 2011;Pereira et al., 2014;Wisse et al., 2014;de Flores et al., 2015b;Daugherty et al., 2016), for a review see (de Flores et al., 2015a)). These discrepancies most likely reflect, at least in part, differences between segmentation protocols. For example, the seemingly disparate findings of age differences in CA1 or subicular volume, might in fact pertain to the same region as these labels often overlap between segmentation protocols.

Hypothetically, new and existing data could be analyzed with the harmonized protocol and not only accommodate direct comparisons of age effects, but also evaluate differences in population characteristics and health (e.g., age-related decline in cardiovascular health (Shing et al., 2011;Bender et al., 2013)) that might account for variability among studies. In addition, a harmonized protocol will

enable the synthesis and integration of results across studies that have included different age groups, thus allowing for a characterization of hippocampal volume across the lifespan without a single lab having to collect data from each age group.

Example 2: Pattern separation and completion

High-resolution fMRI studies of hippocampal subfields aim to elucidate their functional role across various cognitive tasks. Two important mechanisms thought to be involved in human memory and spatial navigation are pattern separation and pattern completion (Marr, 1971;McClelland et al., 1995;Yassa and Stark, 2011). Whereas it is proposed that pattern separation plays an important role whenever similar memories have to be encoded in a distinct fashion, pattern completion is critical for recalling episodes based on partial or degraded cues (Yassa et al., 2010;Hunsaker and Kesner, 2013). Anatomical evidence from non-human animal work, suggests that pattern separation relies on the DG (Leutgeb et al., 2007), while pattern completion appears to be associated with a network of recurrent collaterals in CA3 (Neunuebel and Knierim, 2014).

So far, most human neuroimaging studies of pattern separation and completion mechanisms combine CA2, CA3 and DG into one region, due to limited spatial resolution currently available at 3T (e.g. (Bakker et al., 2008;Yassa et al., 2010;Dudukovic et al., 2011;Kyle et al., 2015b;Stokes et al., 2015)). With new technological advancements at 3T and increasing availability of high-resolution 7T imaging (Feinberg and Yacoub, 2012;Todd et al., 2016), it will become increasingly feasible to functionally separate these subregions. However, most extant segmentation protocols propose different locations of the CA3 boundaries and as a result variable numbers of CA3 voxels are counted towards DG (e.g. (Wisse et al., 2012;Winterburn et al., 2013;Iglesias et al., 2015;Yushkevich et al., 2015b)). This results in high anatomical variability in DG and CA3 masks among protocols. A harmonized protocol will therefore be

critical to better understand the specific functional roles of DG and CA3 in pattern separation and completion using human *in vivo* imaging.

Summary

In summary, we are proposing to develop a harmonized segmentation protocol for *in vivo* T2-weighted MR images for hippocampal and parahippocampal subregions that maximally represent the underlying MTL anatomy while affording high reliability of its measurement and that can be applied in different study populations and on scans acquired in different research groups with different scanners. The development of this harmonized protocol for the currently available 3T and 7T data is crucial in the immediate future as the heterogeneity in protocols is greatly hampering progress of research related to these MTL regions. We believe the current plan will allow us to achieve our main aim of harmonization while also attaining acceptable anatomical validity. However, in the future, the protocol may be updated based upon advances in imaging and analysis techniques, as well as more comprehensive histology reference sets from different populations as they become available via ongoing research endeavors. Additionally, in the future, it would be helpful to obtain a common understanding of the parcellation at the microscopic scale, in which discrepancies in labeling are also an issue, e.g., with CA4 part of some histological segmentations but not others (e.g. (Duvernoy et al., 2005;Insausti and Amaral, 2012)).

We have started our effort by developing a manual segmentation protocol of the hippocampal subfields within the hippocampal body for T2-weighted 3T images because the body is included in all existing segmentation protocols (Yushkevich et al., 2015a) and is more uniform than the head and tail regions of the hippocampus. We plan to extend the protocol to the head and tail portions. We aim to finalize and publish the harmonized protocol for the hippocampal body in the near future to allow for its more immediate adoption while continuing the efforts for procedures in the remainder of the hippocampus, as well as the adjacent cortical regions.

To facilitate wide adoption of the harmonized protocol by new users, we plan to provide training resources, for example an instructional video or an example dataset, alongside the segmentation protocol. Additionally, we plan to implement it into (semi-)automated segmentation algorithms. All extant (semi-)automated segmentation procedures are atlas-based and can be updated with the harmonized segmentation protocol. This step will therefore follow after the initial development of a manual harmonized segmentation protocol. When finished, this manual protocol will be made available to the research groups involved in the development of automated subfield segmentation algorithms (Pipitone et al., 2014; Iglesias et al., 2015; Yushkevich et al., 2015b), several of whom are part of this collaborative effort.

Acknowledgements

We thank the members of the larger Hippocampal Subfields Group for their input on the harmonization effort and insightful comments over the years. We acknowledge the work of Ms. Mercedes Iñiguez de Onzoño for magnificent technical work.

See Supplementary Material for a list of collaborators in the Hippocampal Subfields Groups.

Accepted Article

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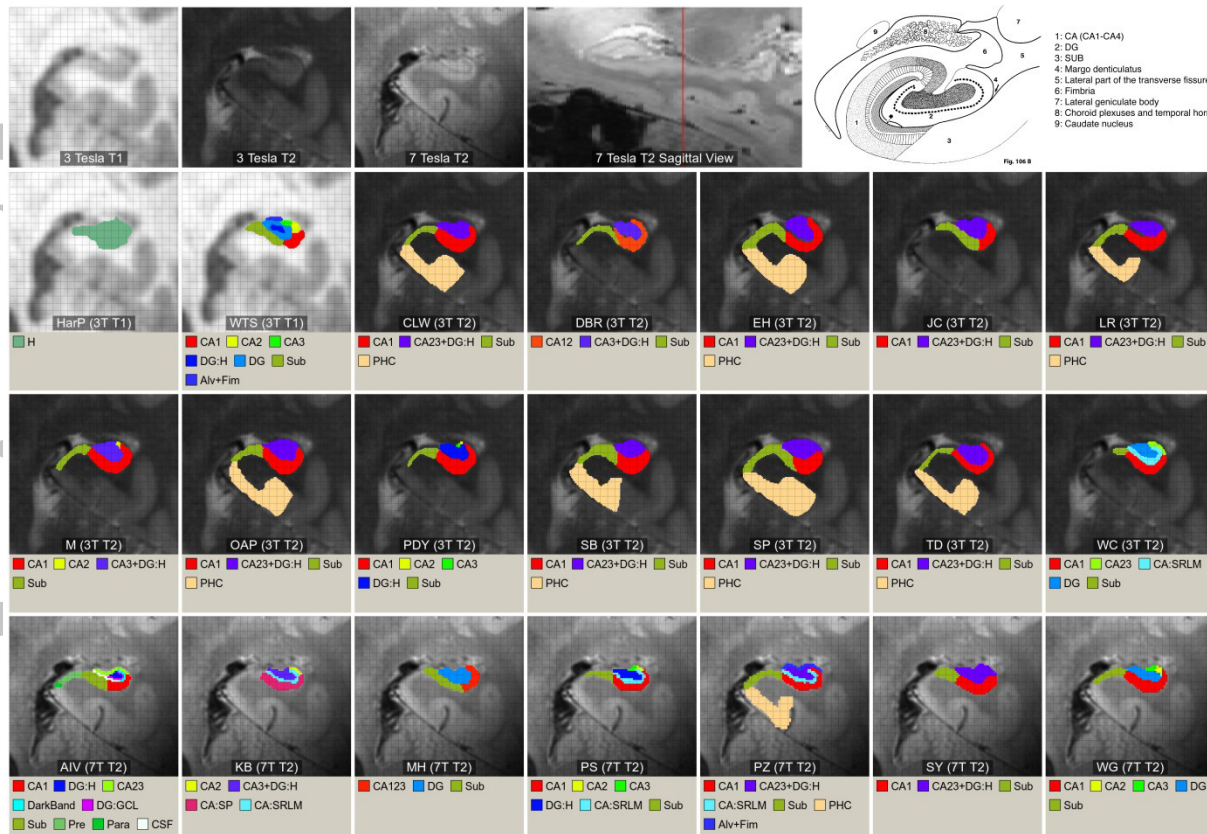
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Figure 1. Comparison of 21 segmentation protocols in a coronal section in the hippocampal body of one subject. Figure from Yushkevich et al, NeuroImage, 2016; reprinted with permission from Elsevier.



Alv=Alveus; CA=Cornu ammonis; CSF=Cerebrospinal fluid; DG=Dentate gyrus; DG:H=Dentate gyrus Hilar region; Fim=Fimbria; GCL=Granular cell layer; H=Hippocampus; Para=Parasubiculum; PHC=Parahippocampal cortex; Pre=Presubiculum; Sub=Subiculum; SP=Stratum pyramidale; SRLM=Stratum Radiatum Lacunosum Moleculare

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Figure 2. Overview of the concrete steps involved in the harmonization project. In Step 1 in a set of three tissue samples, the boundaries on histology will be annotated by three anatomists. In Step 2 the boundaries on MRI will be derived based on the anatomical reference set in Step 1. These boundaries are divided into two sets: the outer boundaries with surrounding structures and the boundaries between the subfields. Note for Step 2 that for both protocols an initial reliability test will be performed and the protocols will be shared with the Boundary Working Group (BWG). In case the reliability criteria are not met or in case of considerable critique, the protocol will be adjusted. This will be an iterative process. In Step 3 feedback will be elicited from the larger Hippocampal Subfields Group (HSG). In Step 4 a formal reliability analysis will be performed by six raters from different labs.

