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Automated Analysis of NeuronJ Tracing Data

Jonathan Popko,[1†] Adelaide Fernandes,[1†] Dora Brites,[2] Lorene M. Lanier[1*]

[1]Department of Neuroscience, University of Minnesota, Minneapolis, Minnesota

[2]Centro de Patogénese Molecular—UBMBE/iMed.UL, Faculdade de Farmácia, University of Lisbon, Lisbon, Portugal

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[†]Authors contributed equally to this work.

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*Correspondence to: Lorene M. Lanier, Department of Neuroscience, 6-145 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455

Email: lanie002@umn.edu

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Abstract

Studies of neuronal differentiation *in vitro* often involve tracing and analysis of neurites. NeuronJ (Meijering et al., Cytometry Part A 2004;58A:167–176; http://www.imagescience.org/meijering/software/neuronj/) is a program that can be used for semi-automated tracing of individual neurons; when tracing is completed, a text file containing neurite length measurements is generated. Using cultured hippocampal neurons, we have found that to reach statistical significance it is generally necessary to trace about 100 neurons in each treatment group. Posttracing data analysis requires importing each text file into a statistics program. Analysis of distinct parameters, such as effects of a treatment on axonal versus dendritic branching, requires a great deal of time consuming posttracing data manipulation. We have developed XL_Calculations, a Java-based program that performs batch analysis on NeuronJ measurement files and automatically makes multiple calculations, including the number, length, and total output (sum length) of primary, secondary, and tertiary neurites on axons and dendrites, and writes the calculations into an Excel worksheet. Batch processing of NeuronJ measurement files dramatically reduces the time required to analyze neuronal morphology. In addition, our program performs more than 45 distinct calculations, enabling detailed determination of treatment effects on neuronal differentiation. Using this program to analyze NeuronJ tracing data, we demonstrate that continuous exposure of differentiating hippocampal neurons to Netrin 1 increases the number of secondary branches on both axons and dendrites, without significantly altering the length of the axon, dendrites, or branches. Similar results were obtained when neurons were grown on poly-D-lysine or laminin.

Key terms

neurite tracing; NeuronJ; ImageJ; axon guidance; growth cone; netrin; laminin

STUDIES of neuronal development and regeneration often use primary neuronal cultures or neuronal cell lines to test the effect of a given molecule on the formation, elongation and branching of axons and dendrites. Typically, cultures are analyzed by immunofluorescence and the number and lengths of the neurites are quantified by tracing of the fluorescent images. Tracing can be done manually using common image analysis software [e. g. ImageJ (1), Adobe Photoshop™], but this is very labor intensive. Fully automated programs are available [e.g. Neurolucida (2) or HCA-Vision (3,4)] and are much less labor intensive, but are often cost prohibitive. An alternative is to use the semiautomated tracing program NeuronJ.

NeuronJ (5) is distributed as a plugin for ImageJ, free image analysis software distributed by the National Institutes of Health (1). Semiautomated tracing with NeuronJ has been shown to be as accurate as both fully manual (5) and fully automated tracing (3). Using NeuronJ, fluorescently labeled neurons are manually traced, but an algorithm compares the pixel intensity on the neurite with its adjacent pixel neighborhoods, automatically updating the cursor to follow an estimated path of the neurite and increasing the accuracy and speed of tracing. After the neurites are traced, a text file is generated that contains measurements of the lengths of all the neurites.

In a typical experiment to determine the effects of a given treatment on neurite outgrowth, we find it is necessary to trace about 100 neurons in each treatment

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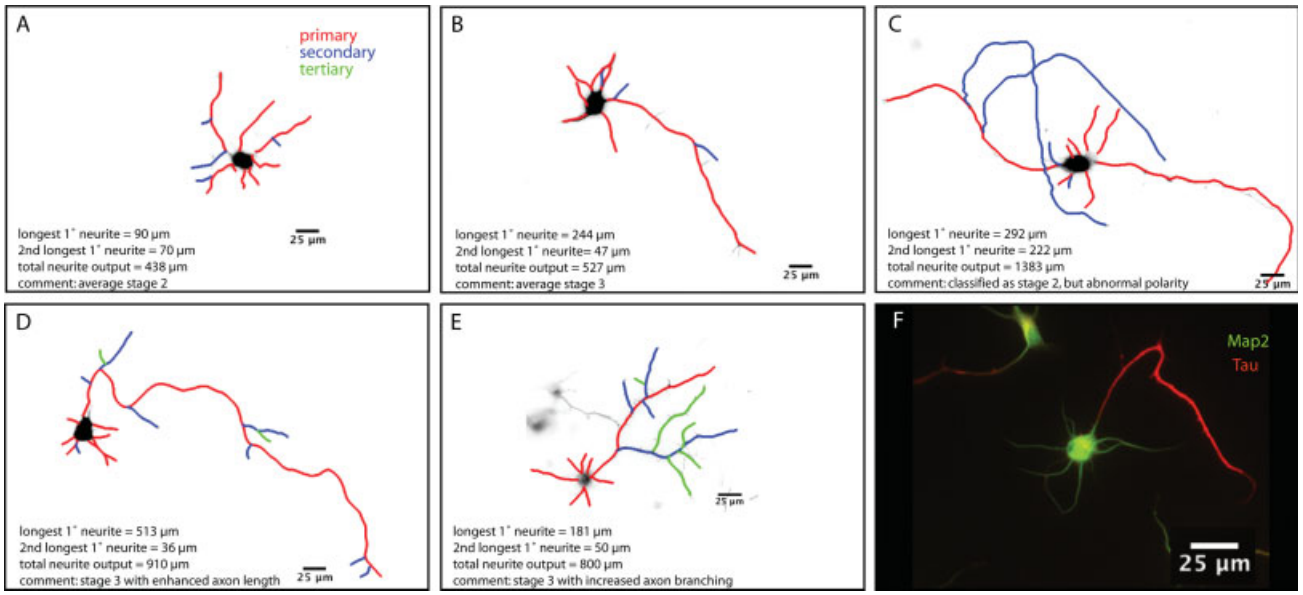

Figure 1. Analysis of NeuronJ tracing data reveals altered neuronal morphologies. (**A**–**E**) Snapshot images of NeuronJ tracings of hippocampal neurons after ~72 h in culture. Images show NeuronJ tracings overlaid on fluorescent images of neurons expressing EGFP. Fluorescent images are shown in black and white and inverted for clarity. Tracings are color coded: red = primary neurite (emanating directly from the soma), blue = secondary neurite (branching from a primary), green = tertiary neurite (branching from a secondary). Lengths of the longest and second longest neurites and the total neurite output are given for each neuron. (A) A Stage 2 neuron with neurite lengths near the average for the population. (B) A Stage 3 neuron with an axon length near the population average. (C) A neuron with two long primary neurites, indicative of a polarity defect. (D) A Stage 3 neuron with unusually long axon. (E) A Stage 3 neuron with reduced axon length, but increased total neurite output due to enhanced branching. (**F**) A Stage 3 neuron stained with antibodies to Tau and MAP2. Tau is enriched in the axon and MAP2 is enriched in the dendrites.

group to obtain statistical significance. Thus, for a simple experiment with a single treatment and control, at least 200 measurement files would be generated. To analyze the morphology of individual neurons, the user must import each measurement text file into a database program such as Excel for statistical analyses. Because of all the manipulations involved, this postmeasurement analysis can be tedious and error prone.

Here we report the development of a software that performs batch analysis on NeuronJ measurement files and makes multiple calculations for a typical cell culture experiment, including the number, length, and total output (sum length) of primary, secondary, and tertiary neurites. These data are subsequently exported to a single Excel worksheet. Use of this program dramatically reduces the amount of time required to analyze NeuronJ tracing data and facilitates analysis of multiple morphometric parameters.

We have used the XL_Calculations program to analyze the effects of Netrin 1 on the differentiation of embryonic hippocampal neurons and report that treatment with Netrin 1 increases the number of axonal and dendritic branches, without significantly changing total neurite output. In addition, similar results were obtained when neurons were grown on poly-D-lysine (PDL) or laminin, indicating that, unlike the growth cone guidance effects, the effects of Netrin 1 on branching are not substrate dependent.

Methods

Hippocampal Cultures and Immunofluorescence

Hippocampal cultures were prepared from embryonic day 16 mice as previously described (6) and plated on coverslips coated with either 1.0 mg/ml PDL or a mixture of 0.1 mg/ml PDL and 4 µg/ml laminin. Netrin 1 (R&D Systems, Minneapolis, MN) was applied at 250 ng/ml and left on continuously. For cultures analyzed in Figure 1, replication-deficient recombinant adenoviruses were produced using the AdEasy system (7) and were used to express enhanced green fluorescent protein (EGFP) in a subset of the neurons. After 72 h, cultures were fixed for 30 min at either 37°C or 4°C in PPS [4% paraformaldehyde in PHEM buffer (60 mM PIPES pH 7.0/25 mM HEPES pH 7.0/10 mM EGTA/2 mM $MgCl_2$) with 0.12 M sucrose]; the 4°C fix was used for staining with Tau and MAP2, and the 37°C fix was used for all other cultures. After rinsing in PBS, coverslips were incubated in 3% fatty acid free bovine serum albumin (BSA) in PBS for 30 min, permeabilized for 10 min in 0.2% triton/PBS, rinsed, and reblocked in 3% BSA/PBS for 30 min. Primary antibodies included anti-Tau (Millipore, Danvers, MA), anti-MAP2 (Covance, Denver, PA), and anti-Tubulin Beta3 (Promega, Madison, WI). Incubations with primary and secondary antisera were done in the presence of 1% BSA/PBS and coverslips were mounted with 2.5% 1,4-diazabicyclo-[2.2.2]octane/150 mM Tris pH 8.0/30% glycerol to

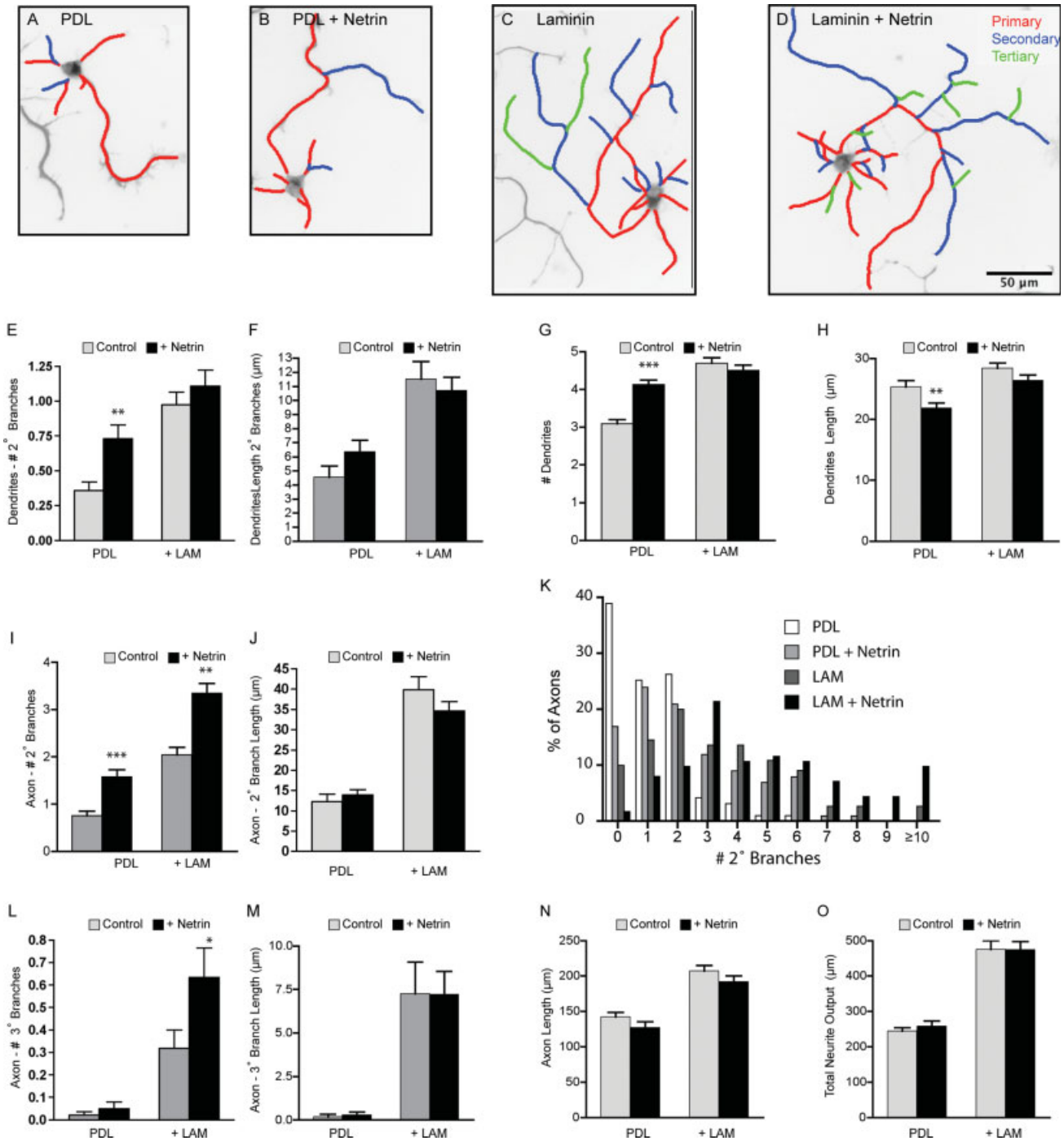

Figure 2. Effects of Netrin 1 on axonal and dendritic branching in hippocampal neurons grown on poly-D-lysine (PDL) or laminin. Examples of neurons grown for 72 h on PDL alone (**A**, **B**) or on a mixture of PDL/laminin (**C**, **D**) in the absence (A, C) or presence (B, D) of Netrin 1 (250 ng/ml). Images show NeuronJ tracings overlaid on fluorescent images of neurons double labeled for Tau and MAP2. Fluorescent images are shown in black and white and inverted for clarity. NeuronJ tracings are color coded for primary (red), secondary (blue), and tertiary (green) neurites. The effects of Netrin I on dendritic (**E**–**H**) and axonal (**I**–**N**) elongation and branching were analyzed in detail for Stage 3 neurons (i.e. neurons with an identifiable axon) and total neurite output was determined (**O**). All treatments: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from Student's *t* test comparing control and +Netrin for each matrix. $n \geq 95$ neurons.

reduce photo bleaching. Cells were imaged on a Zeiss Axiovert 200. Neurites were identified by staining with EGFP and anti-Tubulin Beta3 (Figs. 1A–1E) or with a combination of anti-MAP2 and anti-Tau (Figs. 1F and 2A–2D).

NeuronJ Tracing and Labeling

Neurites were traced using NeuronJ following the developers' instructions. After the neurites are traced, they were labeled as primary (emanating directly from the soma),

secondary (branching from a primary), or tertiary (branching from a secondary). The neurites could be labeled as axon or dendrite; however, assignment of the axon/dendrite labels requires the use of fluorescent markers that are specific to axonal and dendritic regions within the cell. Since many users are tracing neurons that are either labeled with an antibody to neuronal specific Tubulin Beta3 (which labels all neurites) or expressing a fluorescent protein such as EGFP, it is not possible to accurately assign axon and dendrite labels based on staining. Therefore, our program uses the primary/secondary/tertiary labels to perform calculations.

Once labels were assigned, the neurite tracings appeared color coded by type (Figs. 1A–1E). Neurites were then assigned to clusters, with each cluster comprised of a primary neurite and all its associated branches. After tracing was completed and clusters had been assigned, a text file containing neurite length measurement data was generated for each neuron traced and a snapshot of the tracings overlaid on the neuron was saved as a TIFF file (e.g. Figs. 1A–1E and 2A–2D). For the analysis Table 2, 124–145 neurons from each treatment group were traced. Because some of these were Stage 2 neurons, the analysis of Stage 3 neurons (Fig. 2) was performed on 95–112 neurons.

Requirements for Use of the XL_Calculations for Morphometric Analysis

The program was created with Java Development Kit version 1.5.0_13 and requires installation of Java version 1.5 (http://java.sun.com/) or higher on the native operating system and the third party driver Jxl for Excel (http://jexcelapi.sourceforge.net/). This driver is required for Java Database Connectivity import and is included in our software package. The XL_Calculations program also requires Excel from Microsoft Office version 2003 (Windows) or 2004 (Mac) or later and has been tested on both the Mac and Windows versions of Excel. The XL_Calculations program, source code, manual, and parameter customization are available upon request.

The XL_Calculations program is installed by copying a folder containing the Jar program and an Excel template file onto the computer. The program performs arithmetic calculations on the NeuronJ measurement data and writes the calculations directly into an Excel worksheet, with measurements for each neuron in a different row. Detailed step-by-step instructions for using the XL_Calculations program is provided in Supporting Figure 1 and Supporting Methods.

Results and discussion

Using XL_Calculations for Morphometric Analysis of Neuronal Differentiation

The steps required to run the XL_Calculations program are outlined in Supporting Figure 1 and detailed in the Supporting Methods. The XL_Calculations program performs more than 45 different calculations pertaining to the length and branching of neurites (Table 1). The calculations performed by our program are designed to distinguish between neurons at different stages of differentiation: Stage 1, filopodia rich lamellapodium with no identifiable neurites; Stage 2, neuron has neurites, but no identifiable axon; Stage 3, neuron has a rapidly elongating axon; Stage 4, dendrites elongate; Stage 5, synapse formation (8). Stages 2 and 3 neurons can be distinguished biochemically and morphologically (8). The XL_Calculations program uses established morphometric criteria (9) to determine if a neuron is Stage 2 or 3; a neuron is defined as Stage 3 if its longest neurite is at least twice as long as the second longest neurite (Fig. 1B), otherwise the neuron is classified as Stage 2 (Fig. 1A). Once the stage of the neuron is determined, stage-specific calculations are performed. For Stage 2 neurons, the values are reported for all neurites. For Stage 3 neurons, separate calculations are made for axons and dendrites.

By separating the calculations for Stages 2 and 3 neurons, it is possible to determine if a given treatment differentially affects axonal and dendritic outgrowth. In addition, treatments that affect neuronal polarization (i.e. the transition from Stage 2 to Stage 3) are revealed because they alter the percentage of cells at each stage. A treatment that blocks or delays axon formation will increase the percentage of Stage 2 cells. Sometimes, a treatment results in the formation of multiple axons, and such a situation increases the frequency of Stage 2 categorizations because the longest neurites are not twice as long as the second longest (Fig. 1C); however, comparison of the lengths of the second longest neurites in treatments and the control reveals that both are significantly longer than normal dendrites, suggesting abnormal polarity.

XL_Calculations also determines the number and lengths of secondary and tertiary neurites (i.e. branches) and the total neurite output. When analyzing total neurite output, it is informative to know the developmental stage and the contributions from axons, dendrites, and branches. For example, one treatment may enhance axon elongation (Fig. 1D), while another treatment reduces elongation of the primary neurites, but increases branching (Fig. 1E); these likely reflect different underlying biological mechanisms of action. Thus, the calculations made by our program reveal the nuances in neuronal morphology not evident from the "total neurite output" calculation alone.

While our program is optimized for analysis of Stages 2 and 3 neurons in the first few days of differentiation (generally 2–5 days in culture), it can also be used for analysis of Stage 4 neurons, in which dendrite elongation has begun. When analyzing more mature cultures, the user should empirically establish an average dendrite length for Stage 3 neurons, and then set a threshold for dendrite length that distinguishes Stage 3 and Stage 4 neurons. It is important to note that results based on morphometric analyses should always be confirmed by immunohistochemical methods. For example, staining with antibodies to the microtubule binding proteins Tau and MAP2 can be used to identify axons and dendrites, respectively (Fig. 1F).

Computational Advantages of Using XL_Calculations

The arithmetic calculations made by our program could be performed manually (e.g. average axon length or average number of dendrites) and the values obtained by manual

Table 1. Calculations made by the XL_calculations program

Preliminary calculations	
Length of longest neurite	
Length of 2nd longest neurite	
Number of 1° neurites	
Is the neuron Stage 2 or 3?	
Stage 2 calculations for neurites	Stage 3 calculations for axons and dendrites[a]
Average length of 1° neurite	Number of 1° dendrites
Total Number of 2° branches	Sum length or 1° dendrites
Average number of 2° branches per neurite	Average length of 1° dendrites
Average length of 2° branches	Axon length
Total Number of 3° branches	Total Number of 2° branches
Average number of 3° branches per neurite	Average number of 2° branches per dendrite/axon
Average length of 3° branches	Sum length of 2° branches
Total number of branches (2° + 3°)	Average length of 2° branches
Average number of branches (2° + 3°) per neurite	Total Number of 3° branches
Sum length of all branches (2° + 3°)	Average number of 3° branches per dendrite/axon
Average length of branches (2° + 3°)	Sum length of 3° branches
	Average length of 3° branches
	Total number of branches (2° + 3°)
	Average number of branches (2° + 3°) per dendrite/axon
	Sum length of all branches (2° + 3°)
Stage-independent calculation	
Sum length of all 1° (neurites, axons, dendrites)	
Sum length of all 2° branches	
Sum length of all 3° branches	
Total number of 2° branches	
Total number of 3° branches	
Total neurite output	

[a] Separate calculations are made for axonal and dendritic branches.

calculation and the XL_Calculations program are identical (data not shown); however, XL_Calculations performs multiple calculations on a batch of files, greatly reducing the processing time. For example, in a timed trial, it took ~15 min to manually calculate the average total neurite output from 25 NeuronJ measurement files; this included time to open each file, copy the data into an Excel worksheet, determine the total neurite output for each neuron, and finally determine the average total neurite output for the population. Importantly, this manual calculation did not distinguish Stage 2 vs. 3 neurons. In contrast, the XL_Calculations program made all 45 calculation within 40 s; determining the average total neurite output for Stage 2 vs. 3 neurons was a simple matter of sorting and averaging using the appropriate Excel functions; this entire procedure took less than 2 min.

Validation of the Program: Using XL_Calculations to Analyze the Effect of Netrin 1 on Neuronal Outgrowth and Branching

To validate the usefulness of the XL_Calculations program, we have analyzed the effect of Netrin 1 on the differentiation of cultured embryonic hippocampal neurons. Netrin 1 was first identified as a guidance molecule that attracts commissural axons toward the floor plate of the spinal cord (10). Subsequently, cultured *Xenopus* retinal growth cones were shown to turn toward a gradient of Netrin 1 (11). Interestingly, the attraction to Netrin 1 was converted to repulsion when *Xenopus* spinal cord neurons were grown on laminin (12). This observation lead to the speculation that laminin might reverse other effects of Netrin 1.

Laminin is an extracellular matrix molecule that is found in many regions of the developing CNS, including the hippocampus and spinal cord, but it is not present in most layers of the cortex (13,14). Laminin has been shown to selectively enhance axonal growth and accelerate the development of polarity by several types of CNS neurons, including hippocampal and cervical ganglion neurons in culture (15,16). When cortical neurons are cultured on poly-lysine alone (in the absence of laminin), exposure to Netrin 1 increases the number and length of axonal branches, without significantly changing the length of the primary axon (17). Under similar culture conditions, short-term exposure of hippocampal neurons to Netrin 1 induces the formation of axonal shaft filopodia (17,18).

Here we investigate whether Netrin 1 enhances branching in hippocampal neurons and determine if this effect is altered

Table 2. Percent of neurons at Stage 2 vs. Stage 3

TREATMENT	STAGE 2	STAGE 3
PDL	23.4	76.6
PDL + netrin	31.5	68.5
LAM*	11.3	88.7
LAM + netrin*	12.5	87.5

*$P < 0.05$ compared with PDL alone, Student's t test; $n \geq 124$ neurons.

by growth on laminin. As previously shown (15), growth on laminin significantly increased the development of polarity, as reflected by the increased percent of Stage 3 neurons on laminin compared with PDL alone (Table 2). Unexpectedly, treatment with Netrin-1 appeared to slightly delay development of polarity for neurons grown on PDL alone, but not for neurons grown on laminin (Table 2). Because of the relatively small number of Stage 2 neurons at this time point (72 h *in vitro*), further analysis focused on Stage 3 neurons with identifiable axons and dendrites. For Stage 3 neurons, growth on laminin increased the number of dendrites (Fig. 2G), axon length (Fig. 2N), and the number and length of dendritic and axonal branches (Figs. 2E and 2F and 2I–2M), resulting in an increase in total neurite output compared with neurons grown on PDL alone (Fig. 2O).

When neurons grown on PDL were treated with Netrin 1, there was a significant increase in the number of secondary branches on dendrites (Fig. 2E) and axons (Fig. 2I) and a slight, but not statistically significant, increase in the length of secondary branches. Treatment of neurons grown on laminin with Netrin I also resulted in increased branching; however, on laminin only the increase in axonal branches was statistically significant [Figs. 2I and 2L; tertiary dendritic branches are not reported because there were too few (<0.05 3° branches/dendrite) for statistical analysis]. On both PDL and laminin, Netrin 1 increased the frequency of neurons with multiple secondary axonal branches (Fig. 2K).

Surprisingly, on both PDL and laminin, treatment of neurons with Netrin 1 resulted in a slight decrease in the length of primary dendrites (Fig. 2H) and axons (Fig. 2N) and decreased the length of secondary branches on laminin (Figs. 2F and 2J). Although the decrease in neurite lengths was not always statistically significant, it apparently balanced the increase in the number of branches, resulting in no net increase in total neurite output in Netrin 1 treated neurons compared with controls on either PDL or laminin (Fig. 2O).

Together, our results indicate that, contrary to predication, laminin does not block the ability of Netrin 1 to induce branching of hippocampal neurons. In addition, we demonstrate for the first time that treatment with Netrin 1 increases dendrite branching. The observation that the effects of Netrin 1 were more significant on PDL than on laminin may indicate that, on laminin, the neurons are already close to maximizing their neurite output. It is interesting to note that in hippocampal neurons, Netrin 1 does not appear to significantly alter branch length or total neurite output, whereas in cortical neurons, Netrin 1 increases both branch length and total neurite output (17). These observations suggest that Netrin 1 may differentially regulate arborization of cortical and hippocampal neurons *in vivo*.

Conclusion

Use of the XL_Calculations program with NeuronJ greatly reduces the time required to analyze tracing data; analysis of a batch of 50–100 measurement files takes only a few minutes. These calculations can distinguish among treatments that differentially alter neuronal polarity, growth of primary neurites (i.e. axons and dendrites), and branching of axons and dendrites. We have used these detailed calculations to reveal the differential effects of exposure to Netrin 1 on hippocampal axonal and dendritic outgrowth and branching. It is our hope that by facilitating these calculations, our program will make it easier for other investigators to perform a sophisticated analysis of multiple morphometric parameters pertaining to neuronal differentiation.

Acknowledgments

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