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DISCUSSIONS

The Case Western Reserve University Undergraduate Research Journal

FEATURING

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Benjamin Kubit

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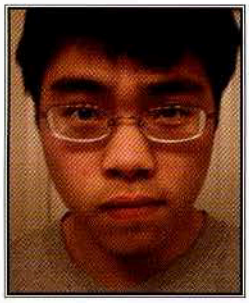

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Letter from the Editors

Dear Reader,

We are excited to celebrate the new year and the 7th year of *Discussions* by presenting to you a new journal filled with student research. As you may have noticed, our schedule has changed over the past semester. Now, the latest issue will be distributed as soon as you arrive back to campus from either the summer or winter holiday, instead of at the end of the semester.

This accomplishes a number of goals. Most importantly, it gives us another three to four weeks of time during the semester, allowing us to push back the submission deadline and spend more time editing the journal. This will hopefully lead to an even higher standard of quality for future journals. Because of the extra time for submissions at the beginning of the semester, we will be doing away with the multiple deadlines and extensions of semesters past, so if you have an article to submit for next semester, take note of the one and only deadline, February 14th.

The change in schedule also allows us to coordinate distribution and advertising of the new issue with advertising for submissions for the next issue at the start of the semester. Previously, journals were quietly released to a campus with little time to do anything but study for finals. We think fellow students will have more time and interest at the start of a fresh semester.

Lastly, we encourage all of the students reading this to consider joining us on the review board! We welcome students of every background and experience level. The board meets twice a semester to eat and discuss a batch of submissions. If you have more time available, you're welcome to join the advertising, editing, and/or design boards. Come learn new skills and gain new experiences! Just to sweeten the deal, executive members receive a small yearly stipend for their work.

As usual, send your submissions, questions, or opinions to discussions@case.edu.

Cheers,

Sean Yeldell, Editor-in-Chief
Bharathi Muthusamy, Asst. Editor-in-Chief

New Plug Designs for Improved Mechanical Properties in Al 2195 Friction Pull Plug Welds

Rachel Craft

Rachel Craft is a third-year materials science and engineering major with a minor in English and Math. She is from Bloomington, IN and works as a copy editor and reporter for The Observer. Her hobbies include art, writing, belly dancing, and martial arts. She is also active in intramural sports, the Undergraduate Materials Society, the Case Animal Rights and Ethics Society, and the Alpha Chi Sigma professional chemistry fraternity. This paper was written as part of a summer internship at the NASA Marshall Space Flight Center, where she explored her interests in metallurgy and aerospace applications.

-Acknowledgments-

I would like to thank the NASA Marshall Space Grant Research program for funding this project, as well as the Marshall Space Flight Center for providing resources and facilities. I would also like to thank my mentor, Shane Brooke, and the numerous coworkers who provided contributed to the research: Brad Anders, Steve Cato, Greg Neal, Bobby Greenhill, Corey Sloan, John Swieder, and Vann Bradford.

ABSTRACT

Friction pull plug welding (FPPW) is a relatively new process that has already found applications in the aerospace industry. FPPW is based on many of the same principles as friction stir welding (FSW), and is primarily used to close out or repair circumferential self-reacting friction stir welds (SR-FSWs) of aluminum alloys such as Al 2195. Although FPPW has been shown to be effective, one important disadvantage is that the resulting welds tend to be weaker than the surrounding FSW. Research was concentrated on improving the mechanical properties of pull plug welds by altering the shape of the plugs used. Three new Al 2195 plug designs—stepped head, left-hand threaded head, and right-hand threaded head—were welded onto SR-FSW test plates, then examined under microscope to observe the resulting flow patterns, microstructures, and bond line characteristics. Specimens underwent nondestructive evaluation (NDE) to assess weld soundness and the presence of defects; they were also mechanically tested to determine their ultimate tensile strength (UTS). The resulting data found the right-hand threaded plugs to be disadvantageous, but the left-hand threaded and stepped plugs appeared promising. More research is in progress to further develop their designs.

INTRODUCTION

Friction stir welding (FSW) is a solid-state process which uses mechanically generated heat to plasticize and physically deform, or stir, the weld metal to bond the two sides of a joint (Dickinson, et al.). It presents an attractive alternative to other welding methods because it does not require filler metal, flux, or shielding gas and does not generate sparks, smoke, or fumes. Also, FSW is unique to other techniques because the stirring process aggressively disrupts surface films so rigorous cleaning of the parts prior to welding is not of concern (O'Brien). Because the metal is not melted and recast, FSW joints do not experience many of the problems inherent in fusion welding, such as alloy segregation, grain growth, phase structure alteration, porosity, and solidification cracking (Nunes). The FSW process produces a distinctive microstructure in the center of the weld, or the "weld nugget." Because recrystallization occurs under high temperature and severe plastic strain, the resulting weld nugget microstructure is characterized by fine, equiaxed

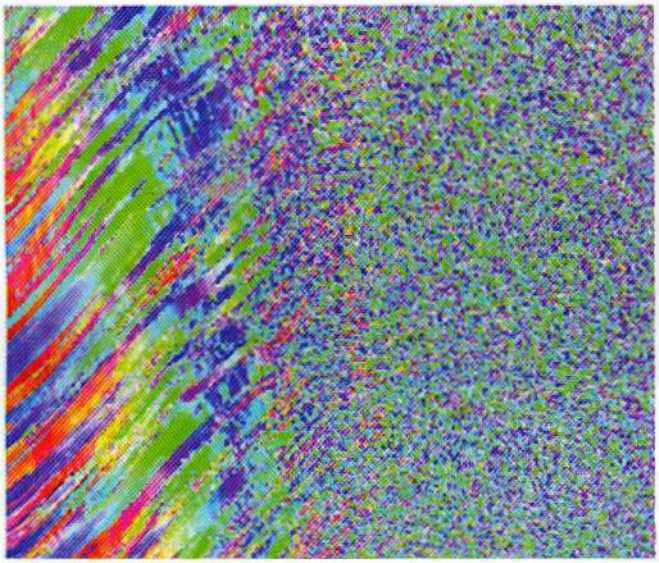


Figure 1. Electron backscatter diffraction/ orientation imaging microscopy image showing the sharp boundary between the parent metal grains (left) and the recrystallized nugget grains (right) (Schneider).

grains, as depicted in Figure 1. Because of these characteristics, FSW joints better retain baseline mechanical properties, experience less distortion, and result in lower residual stresses and fewer defects than fusion welds (Uematsu, Tokaji, Shibata,Tozaki, and Ohmune). FSW is known for producing remarkably sound welds. In fact, it is possible to weld over two miles without a single defect and the weld metal is almost always observed to be at least as strong as the base metal. FSW is applicable to a diverse range of dissimilar metal combinations, although the material of interest in this study was the aluminum-lithium alloy Al 2195 (O'Brien).

When welding a shape such as a dome, conventional FSW techniques need to be modified. The considerable reactive forces involved in FSW require heavy tooling. For practical purposes, a self-reacting pin tool is used to function as both pin tool and anvil simultaneously. This type of pin tool is inserted into a hole in the joint. A root shoulder is screwed onto the end of the pin tool and a crown shoulder is pushed against the opposite side of the metal. During the weld, the pin and root shoulder are pulled up and the crown shoulder is pushed down with equal force while the pin spins rapidly, stirring the plasticized metal just as in a conventional FSW.

This type of FSW is ideal for circumferential welds, such as that between a metal dome and connecting ring. In this case, the pin tool must be removed afterwards, leaving a hole in the weld line. This exit hole is sealed, or "closed out," by means of a relatively novel technique known as friction pull plug welding (FPPW), depicted in Figure 2. A pull plug weld is made by inserting a metal plug into the hole, then spinning it at a very high rate and pulling it through the opening. The remainder of the plug protruding from the weld may then be machined off to yield a flat surface.

The disadvantage to this technique is that no upsetting occurs in the plug material. Upsetting is a phenomenon in which heat generated along the weld line causes the metal along the edge of the plate to be plasticized and extruded out. Upsetting is crucial to the strength of the weld; it forces contaminated surface material out of the weld, exposing clean, fresh metal. Without contaminants such as surface oxides entrapped in the weld, the plug and plate can make intimate contact and a very sound bond. As can be seen in Figure 3, the metal on the edge of the hole is extruded out but the plug experiences little or no deformation. This is likely because deformation is more favorable in the short transverse direction in the plate than in the longitudinal direction in the plug (McClure).

As a result, pull plug welds exhibit relatively little physical mixing and thus rely more on diffusion bonding. Also, the plug's surface material—and any contaminants it may carry—becomes entrapped in the weld. Surface contaminants, such as metal oxides, tend to weaken the weld material. Previous research has shown that plugs subjected to chemical cleaning produce welds with higher ultimate tensile strength (UTS) and lower standard deviation than as-machined plugs (Brooke).

The uncleaned specimens failed directly along the bond line—between the equiaxed grains of the weld and the longitudinal grains of the plate or plug—resulting in a very clean fracture surface, which indicates relatively low strength. The cleaned specimens, on the other hand, show a much rougher fracture surface, suggesting that they failed in the heat-affected zone (HAZ) of the plug weld rather than the weld itself. This type of fracture is more desirable, as the rough surface and large dimples indicate higher plasticity and tensile strength. As a result, chemi-

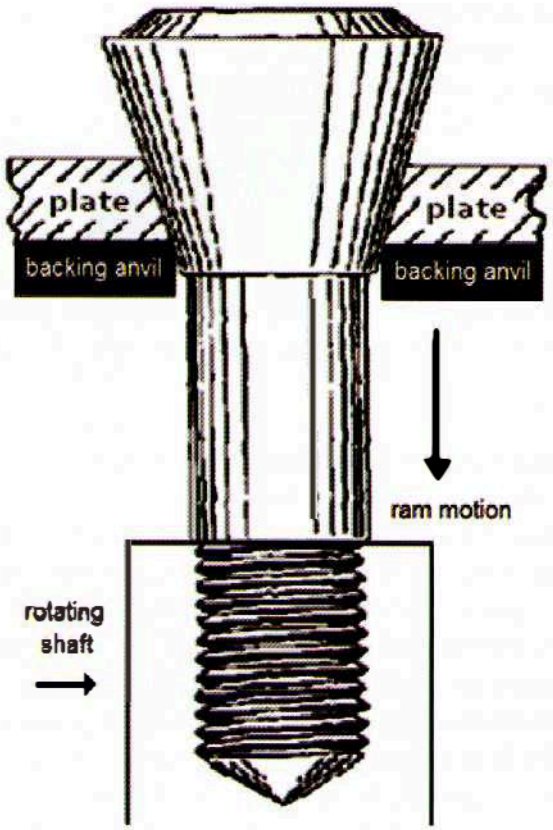


Figure 2. Illustration of FPPW. The plug shank is screwed into the weld tool, then the plug is pulled through the hole while rotating at very high RPM.

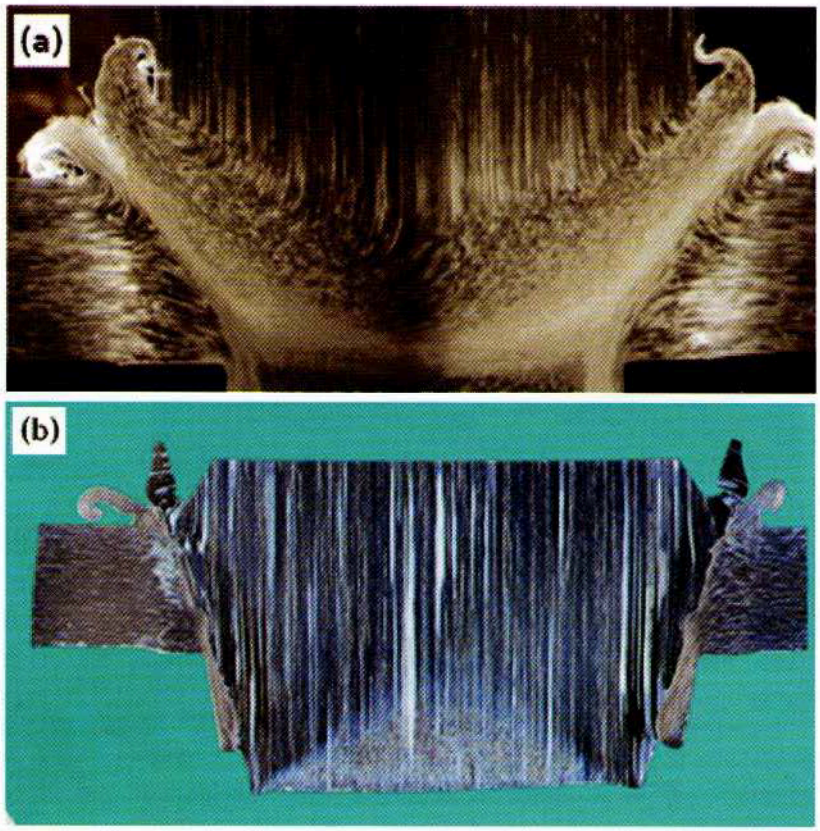


Figure 3. Cross-section of (a) a finished push plug weld, exhibiting upsetting in both the plate and plug material (McClure and Nunes), and (b) a finished pull plug weld, in which the plug experiences relatively little deformation (McClure).

cal cleaning has become a standard procedure in pull plug weld processes (Brooke).

Despite rigorous cleaning methods, some degree of contamination is inevitable. For example, an aluminum surface will develop a layer of aluminum oxide a few nanometers thick within a matter of picoseconds upon exposure to air (Campbell, Kalia, Nakano, Vashishta, Ogata, and Rodgers). The only way to fully circumvent this problem is to induce upsetting in the plug in order to remove contaminated surface material. If the upsetting observed in the parent metal were to occur in the plug metal as well, the surface material and its contaminants would be extruded out, making plug cleanliness far less of a concern. It was theorized that it may be possible to alter the plug design to promote more upsetting. The aim of this project was to develop new plug designs, examine their weld behavior, and analyze the microstructural and mechanical properties of the resultant welds.

Microstructural evaluation is a valuable tool in predicting a weld's behavior in service. Not only can microscopic examination reveal voids and other defects which play a role in weld quality, but it can provide information on the grain size and shape which may also influence mechanical properties. Microscopy may also be used to study the bond line, which is essentially a strip of weld metal between the plug and the plate. The bond line is made up of small, equiaxed, recrystallized grains (just as in FSW), and may play an important role in weld strength, although this role has yet to be fully understood.

Microstructural patterns are excellent indicators of weld soundness, but they only provide fuel for speculation—mechanical assessment is required to know exactly how and under what conditions a weld will fail. There are various ways to determine this. The techniques used in this study were dye penetrant testing and tensile testing. Dye penetrant testing is a form of nondestructive evaluation

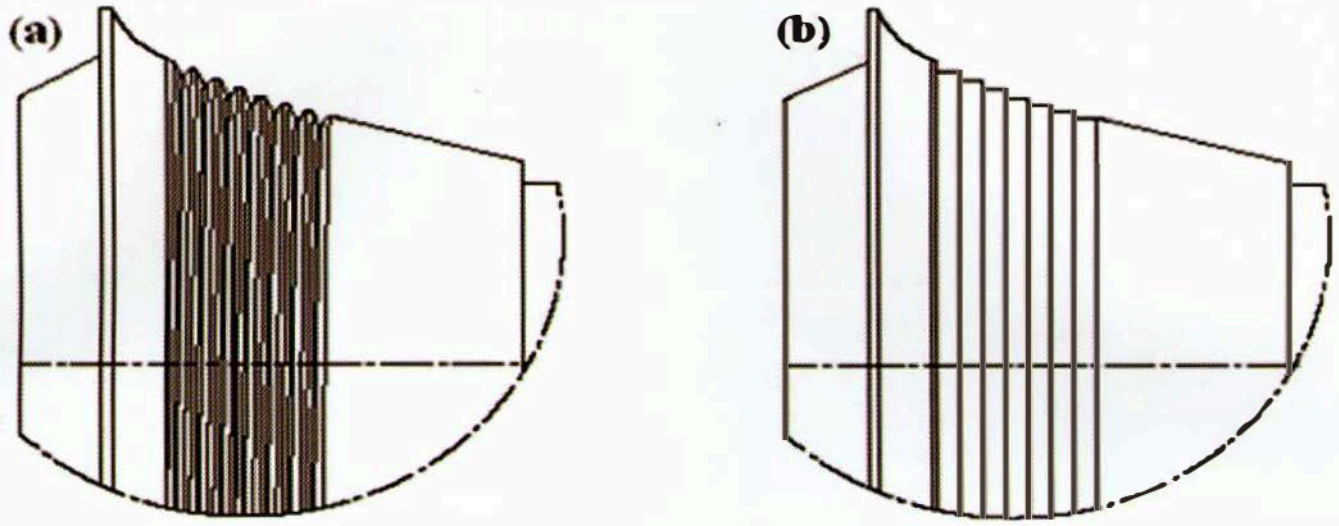


Figure 4. Close-up illustration of the plug head for the (a) threaded and (b) stepped plug designs.

(NDE) in which a fluorescent dye, or penetrant, is applied to the specimen, then by capillary action is drawn into any cracks or voids that are open to the surface. After the excess penetrant is removed, a developing agent is applied to the surface to draw the remaining dye out of any crevices. The dye that bleeds out will be visible under a black light, identifying any defects that are open to the surface. This is an effective way to assess the general bond quality: it will identify any welds that are not fully bonded, as well as any significant voids near the surface.

Tensile testing is used to determine quantitative properties such as UTS. Test panels are cut so that each weld is in the center of a tensile bar (with length perpendicular to the original FSW), which is pulled in tensile from both ends until failure. The stress measured at failure is the UTS, which was of interest because it can give a quantitative measure of which plug design produces the most effective weld.

PROCEDURE

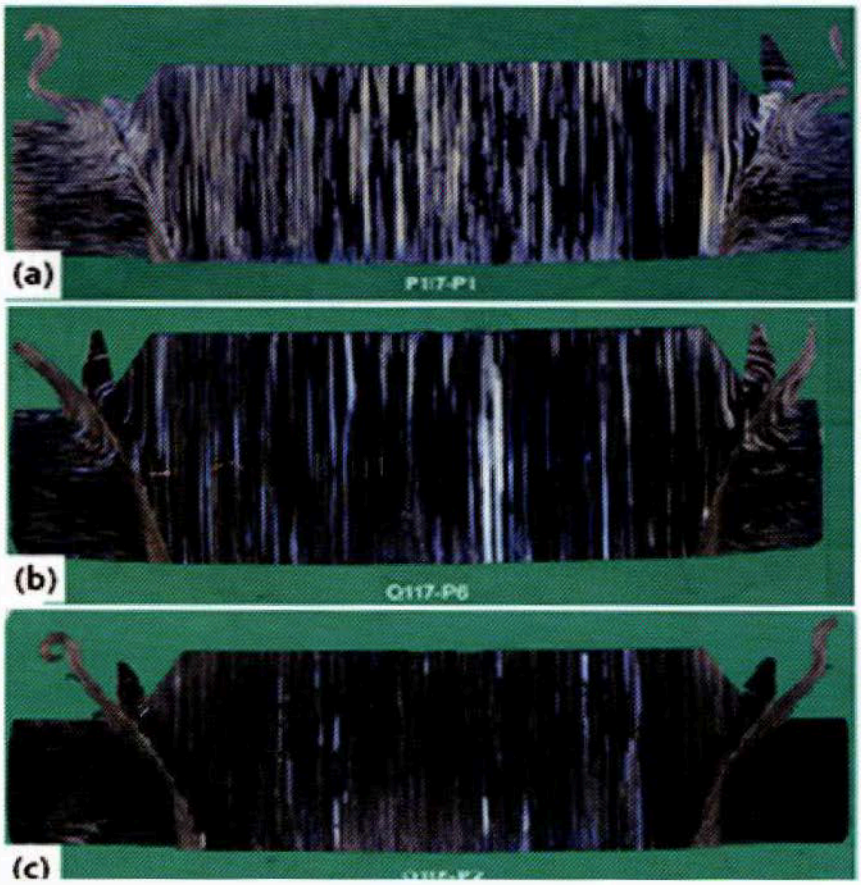
Three new plug designs were proposed: stepped head, left-hand threaded head, and right-hand threaded head (examples are shown in Figure 4). It was hypothesized that removing some material at intervals along the existing plug head might allow for transverse deformation in the plug and help plasticize the plug metal, induce mixing in the weld, and extrude the dirty plug surface material. The small fins on the threaded heads should theoretically help stir the surrounding plasticized material. Additionally, the fins' small thickness should cause them to heat up quickly and be more prone to plastic deformation.

For each of the three new designs, 16 standard Al 2195 plug specimens were machined according to the design specifications. Thirteen of the plugs—five right-hand threaded, four left-hand threaded, and four stepped—underwent an acetone wipe and wire brush scrubbing before being welded onto an SR-FSW joint on a 0.327"-thick Al 2195 test panel using weld parameters for typical plugs of the same size. Each weld was then cross-sectioned, mounted, polished, and etched in preparation for microscopic evaluation.

Following microstructural analysis, 12 more test welds were made to assess the welds' mechanical properties. Four plugs of each design were chemically cleaned, alcohol-wiped, and wire brushed prior to being welded onto 0.327" Al 2195 SR-FSW test panels. These weld specimens then underwent NDE in the form of dye penetrant testing to search for indications. They were then cut into 12 tensile specimens in preparation for tensile testing to determine UTS.

ANALYSIS

Macrographs of representative plugs of each design are



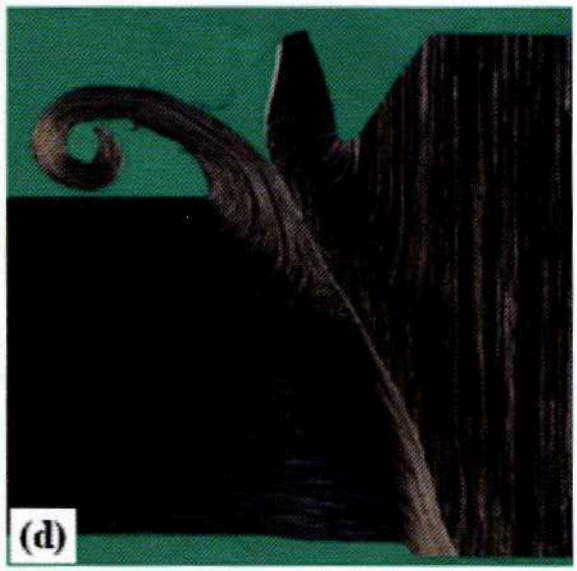


Figure 5. Cross-sections of test welds using (a) left-hand threaded, (b) right-hand threaded, (c) stepped, and (d) standard plug specimens.

shown in Figure 5. None of the test welds showed significant deformation in the plug. In fact, cross-sections show upsetting very similar to that in conventional plug welds: the plate deforms and is extruded from both sides, while the plug exhibits little or no deformation.

The bond lines in the test welds (Figure 6) show a marked change from the smooth, straight line produced by traditional weld plugs (Figure 7). The bond lines produced by threaded plugs have a jagged, shark-tooth shape, while those produced by the stepped plugs are smoother but noticeably bumpy.

The test welds are similar to typical plug welds in that the bond lines each consist of a strip of tiny, equiaxed grains (visible in Figure 6) separating the longitudinal grains of the plug and plate material. The right- and left-hand threaded plugs produced bond lines which are largely straight except for narrow fin-like protrusions. This shape suggests that during the weld, the threads folded in on themselves, forming gaps to be filled by recrystallized grains. The stepped plugs produced a similar, if less pronounced, phenomenon. In this case, the steps collapsed against the side of the plug to form a bond line with periodic bumps. The only voids observed in the test welds were located at the tips of the fins along the bond lines of the threaded plug welds, although these voids were too small to likely influence weld strength. In general, the welds appeared defect-free.

Although mechanical testing is required to determine a weld's strength, speculation can be made based on microstructural observations. According to the micrographs in Figure 6, the test welds have potential: there are no glaring defects, and the plug and plate appear to have bonded just as well as in conventional plug welding. Although the test welds did not exhibit significant deformation and extrusion of plug surface material, their bond line characteristics suggest that the threads and/or steps may still have a positive effect on mechanical properties. The jagged nature of the bond line in the threaded specimens should, theoretically, present a less convenient path for crack propagation between the bond line and the parent grains (as compared to the straight bond line seen in traditional plug welds).

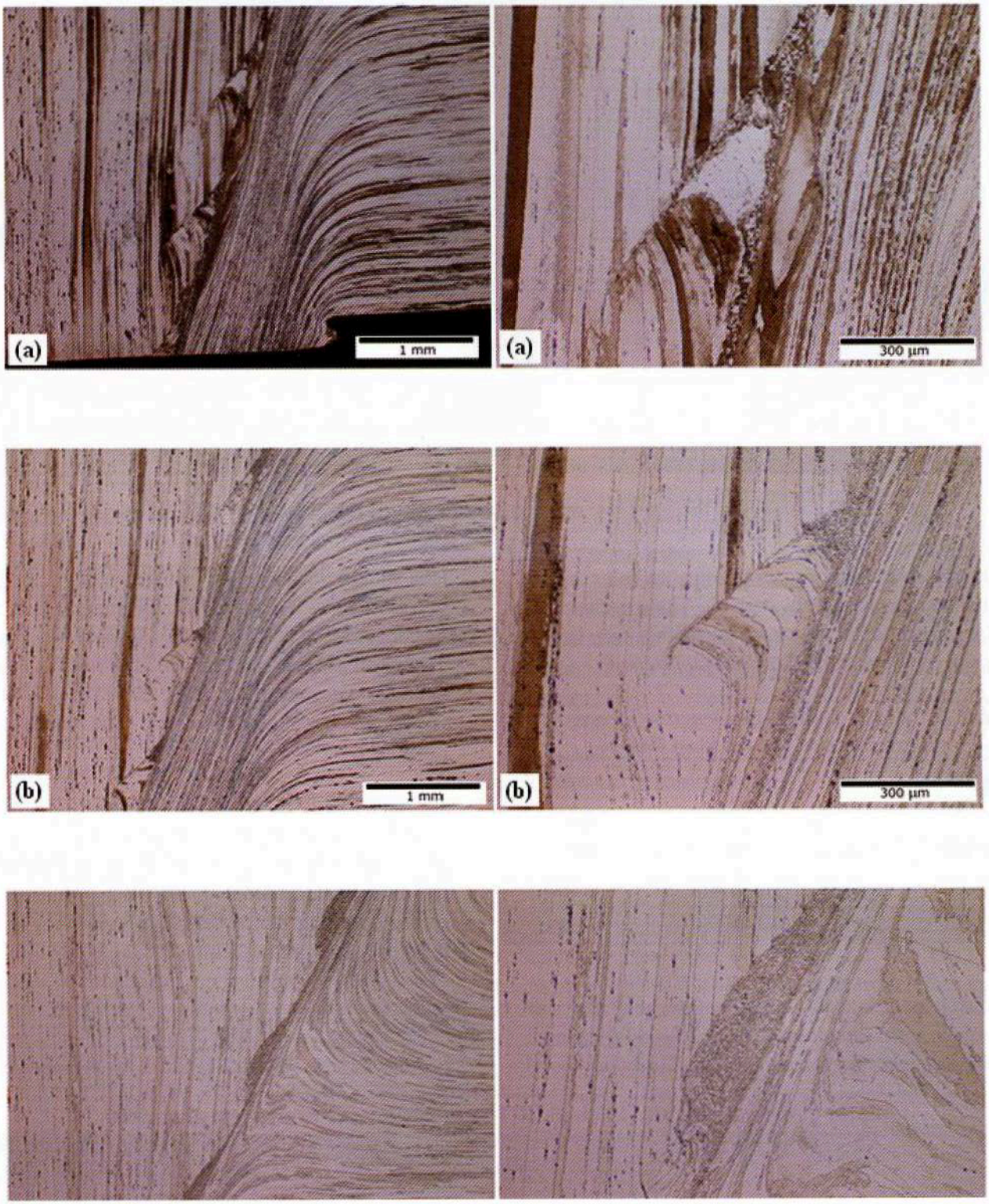


Figure 6. Micrographs of the bond line of (a) right-hand threaded, (b) left-hand threaded, and (c) stepped specimens at both 25 and 100x magnification.

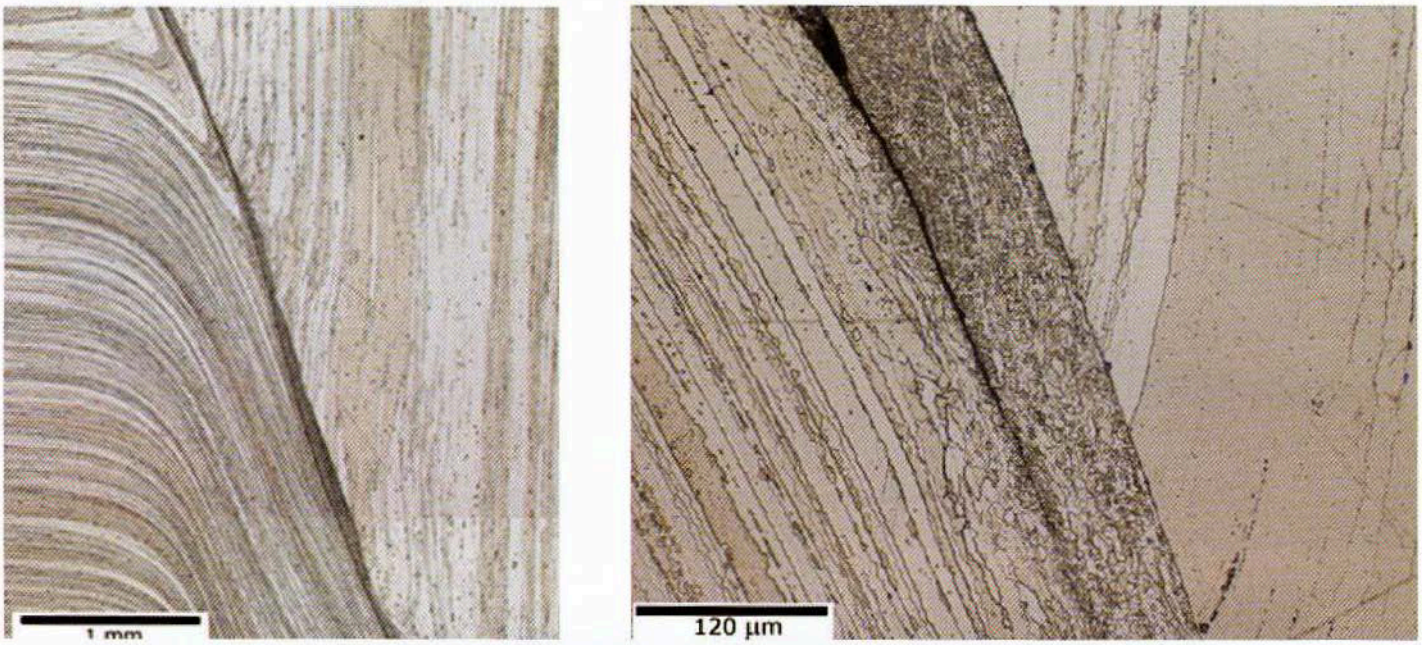


Figure 7. Micrographs of the bond line of a standard plug weld, shown at two different magnifications.

This should discourage fracture along the edge of the bond line and promote weld strength. The wavelike pattern of the bond line in the stepped specimen may have a similar effect, although perhaps less pronounced.

Table 1. Calculated bond line lengths (inches) for representatives of each plug design.

plug ID	design	as-machined	as-welded
Q117-P5	RH thread	0.560	0.687
Q117-P9	LH thread	0.561	0.586
Q118-P2	stepped	0.448	0.400
Q120-P1	standard	0.357	0.380

It was also hypothesized that length of the bond line may play a role in weld soundness: because the weld material is usually stronger than the parent metal, increased bond area should translate to a stronger bond. A jagged bond line is likely to be longer than the straight line exhibited by conventional plug welds. To compare this characteristic, bond line length before and after welding was calculated for representatives of the three types of test welds and for a conventional plug weld. The results are shown in Table 1.

All three test plugs, the threaded plugs in particular, produced a longer bond line than the standard plug. The test plugs also showed a significant change in bond line length during the weld, which theoretically indicates mixing and/or upsetting. Since very little upset was apparent in the cross-sections, this length change is most likely a sign of considerable physical mixing inside the weld, another indication of weld soundness.

For the 12 test welds designated for mechanical assess-

Table 2. Ultimate tensile strength (UTS) values for the twelve test plug welds.

plug ID	design	UTS (ksi)	plug ID	design	UTS (ksi)	plug ID	design	UTS (ksi)
Q118-P5	stepped	58.34	Q119-P1	LH thread	53.11	Q119-P5	RH thread	45.68
Q118-P6	stepped	56.04	Q119-P2	LH thread	53.59	Q119-P6	RH thread	46.58
Q118-P7	stepped	55.64	Q119-P3	LH thread	52.69	Q119-P7	RH thread	47.89
Q118-P8	stepped	53.61	Q119-P4	LH thread	56.02	Q119-P8	RH thread	45.55

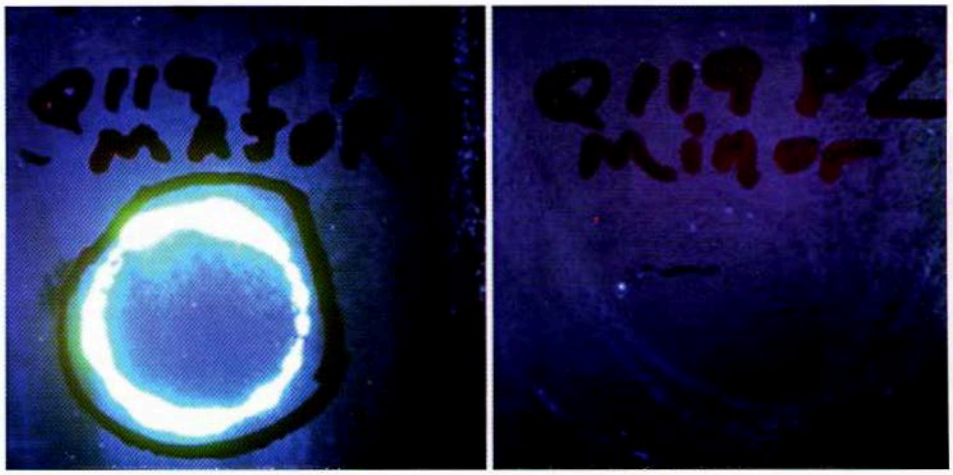


Figure 8. Photographs under black light of the reported indications in (a) Q119-P7 and (b) Q119-P2.

ment, dye penetrant testing was performed on both the crown and root side of plug weldments. The most noticeable NDE result was a linear defect around the circumference of Q119-P7, shown in Figure 8 (a). The significant bleed-out suggests that the plug was pulled in too far during the weld, leaving a large circumferential gap. Another look at the TTTA measurement (the distance from the top of the plate to the top of the plug after welding) verifies this. This is likely due to a control problem with the weld tool, which is now under investigation.

The other test welds exhibited a few small rounded indications. The only one large enough to be reported was observed on the minor side of Q119-P2 (a left-hand threaded plug), at 0.010" diameter, and is shown in Figure 8 (b). The small size and low frequency of these indications suggests that the welds, excluding Q119-P7 and potentially Q119-P2, should be fairly sound.

Table 3. Standard 0.327" 95/95 SR-FSW plug weld data (ksi).

avg UTS	min UTS	max UTS	std. dev.
56.69	55.21	58.28	1.25

Although speculation was made based on microscopy and NDE, tensile testing was required to obtain quantitative strength data. The results are shown in Table 2. The UTS values for the stepped plug heads are consistent with those of standard plug welds (see Table 3). The left-hand threaded specimens were slightly weaker in comparison, and the right-hand threaded plugs showed a significant strength decrease.

DISCUSSION

Because only four plugs of each design were tensile tested, it is impossible to get a reliable range of UTS values for the new plug designs. More tensile tests will be performed in order to determine accurate minimum, maximum, average, and standard deviation values. However, based on the present observations, only the left-hand threaded and stepped plugs will be subject to further study, while the right-hand threaded design will likely be abandoned. The designs may be altered slightly—for instance, by changing the thread pitch or step size—to see if weld properties may be further improved. Tensile testing will also be performed on specimens of different widths in order to determine how strength varies with sample width, and to compare this variation with that of standard plug welds.

Of particular interest are the two welds showing dye penetrant indications. The UTS for Q119-P2, which had a small rounded void, was consistent with the values for other left-hand threaded plug specimens. More importantly, Q119-P7, which showed a glaring linear defect, turned out to have the highest UTS of the right-hand threaded plugs. This suggests that, although the right-hand threaded plugs yielded disappointing tensile results, these plugs may have potential for improved standard deviation. Greater strength consistency is very desirable from a practical standpoint, so this is something that warrants further investigation. Future tensile testing will be performed on left-hand threaded and stepped plugs to determine their potential for improved standard deviation.

The current tensile specimens will undergo fracture analysis, which will hopefully provide a better understanding of how failure occurred. This may help to clarify how the changes to the plug designs affect failure mechanisms and how the plugs may be further altered to improve the mechanical properties of the welds.

Also, the test welds' microstructural and bond line characteristics will be reassessed in light of the tensile test results. Clearly, the speculations made in this study based on bond line analysis were inaccurate. Perhaps bond line length is a poor indicator of weld strength, or perhaps its relationship with weld strength is simply misunderstood. It is also possible that other factors, such as bond line thickness, play a more important role than previously expected. Hopefully the data obtained in this experiment can be used to develop a better understanding of the microstructural factors in plug weld quality.

CONCLUSION

From the tensile test results, the right-hand threaded plugs appear to have little potential for improving weld quality. The left-hand threaded and stepped plugs, however, have promise. Because of the limited number of test welds, it is difficult to get a conclusive idea of the weld strength of the new plug designs; further tensile testing will be conducted to accumulate more UTS data and get a better idea of the strength range for these designs. The fracture surfaces of the current tensile specimens will also be examined to better understand how the design alterations influence failure mechanisms.

Although the right-hand threaded plugs produced disappointing tensile test results, their limited UTS deviation with regards to the sample with a dye penetrant indication is appealing. Future research should focus on determining if the stepped and left-hand threaded plugs show a similar consistency. If so, these designs would be a beneficial alteration to the current weld process, even if their strength values were not significantly higher than those of the standard plug welds. Additionally, further study of the test welds' microstructural characteristics—bond lines in particular—will help develop a more accurate understanding of how these characteristics contribute to weld strength.

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Conformational Dynamics of Wild-Type Neuroserpin in Relation to Human Protein Misfolding Diseases

ABSTRACT

Wild-type human neuroserpin, a member of the serine protease inhibitor superfamily, is expressed in neurons of the central and peripheral nervous system, as well as in the adult brain. Polymerization of certain mutants of neuroserpin is associated with dementia caused by familial encephalopathy, but the protein can also inhibit the toxicity of amyloid-β-peptides in Alzheimer's disease by binding to them[1]. We have performed hydrogen/deuterium exchange mass spectrometry in order to monitor the structural stability and flexibility of different regions of the neuroserpin structure. We found that a critical region thought to be involved in polymerization is less stable and more labile in neuroserpin than in other serpins such as alpha-1 antitrypsin and antithrombin. This may explain why wild-type neuroserpin is more susceptible to polymerization than other serpins. Molecular dynamic simulations of the wild-type neuroserpin and a disease associated mutant were performed in order to probe the molecular motions at atomic level detail. Principle component analysis was then used to interpret the molecular dynamic simulations. Correlation diagrams show that the mutant neuroserpin simulations may have more correlated movements than the wild-type neuroserpin. Furthermore, the mutant showed distortions near the top of the central beta-sheet, a region believed to be a critical site for polymer formation. The distortion could explain why the mutant protein is more likely to polymerize than the wild type protein.

INTRODUCTION

Misfolding of a protein is the cause of many human diseases. In the case of wild-type human neuroserpin, misfolding can cause dementia. Affected individuals show signs of cognitive difficulties including reduced attention and concentration, response regulation difficulties, and impaired visuospatial skills[2]. Mutation of certain amino acids in this protein also leads to diseases such as myoclonus, epilepsy, and chorea as well. Therefore, studying the folding mechanism and the global interactions of wild-type human neuroserpin will be particularly useful in understanding these diseases and then leading to possible treatment.

Crystal Zhou

Crystal Zhou is a senior at Case Western Reserve University studying Biochemistry, with minors in Biology, Chemistry, and Sociology. She has been an active member of the Asian American Alliance and is the President this year. She also serves as the Vice President of Intellectual Development (Scholarship Chair) of her sorority, Alpha Chi Omega, and gives back to the community by participating in various service and philanthropy events with her sorority sisters. Some of her future goals are to continue her research and design drugs that may one day cure cancer.

-Acknowledgments-

I would like to thank R. Meklemburg for his help conducting the various experiments and P.L. Wintrode for his instruction, patience, and guidance throughout this project. I would also like to thank the Summer Program for Undergraduate Research, funded by the Howard Hughes Medical Institute, for allowing me this wonderful opportunity to experience full-time research.

Hydrogen/deuterium exchange measured by mass spectrometry helped us determine if parts of the protein were flexible or stable. Flexibility of the protein was determined by monitoring which peptides exchanged first, and stability of the protein was determined by monitoring which peptides exchanged the least or did not exchange at all. The information from the exchange also allowed us to determine which parts of the protein are exposed to the environment and which parts are folded inside, as well as which regions of the protein are less stable and susceptible to polymerization.

Molecular dynamic simulations showed how wild-type human neuroserpin and a mutant form act at the atomic level. From the simulations, we were able to gather information about the movement of the protein due to its interactions with the environment. Principle component analysis was useful because it allowed us to figure out which regions of the protein experienced correlated movements, the root mean square distance between residues, and the energy levels of the protein by using the first, second, and third principle components. Energy landscape diagrams helped determine which parts of the protein are more stable and change little over time. Our results provide insight into the structural stability and reasons why the mutant form of neuroserpin is more prone to polymerization than the wild-type.

MATERIALS AND METHODS

Purification:

A seed culture of BL21 cells was grown in LB media overnight. The cells were then allowed to grow in 2L of 2XYT media for 3 hours. Cells were then induced to express wild-type neuroserpin by adding IPTG to the media and incubated at 20° C for 17 hours. The cells were then harvested and allowed to incubate in lysozyme and PMSF on ice-water for half an hour. The cells were disrupted by sonication, washed, and incubated in the cold room for 45 minutes with Ni-NTA beads. The Ni-NTA agarose was then washed with Buffer A (50mM sodium phosphate, 10mM imidazole, 500mM sodium chloride, pH 7.8), packed into a column, connected to an FPLC system, and washed again with Buffer A followed by 20mM sodium phosphate buffer, pH 7.8. The protein was eluted with 250mM imidazole, 20mM sodium dihydrogen phosphate, pH 7.8. The eluate was diluted fourfold with Buffer C (20mM Tris-hydrochloride, 20mM sodium chloride, pH 7.4), washed with Buffer C in a Hitrap Q HP and eluted. The final eluate was diluted twofold with Buffer B. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify that a protein was present in the sample. The purified sample of neuroserpin was separated into 2 µL aliquots and kept in the -80 °C storage.

Hydrogen/Deuterium Exchange-Mass Spectrometry:

Seven aliquots were used for this experiment. Each aliquot was diluted 25-fold with D2O Buffer (6.4M Guanidine Hydrochloride, 10mM sodium phosphate, 50mM sodium chloride, pD 7.8) and pulse labeled for either 10, 50, 100, 500, 1000, 2000, or 3000 seconds. After the time point was reached, the reaction was quenched by diluting the sample twofold with 100mM sodium dihydrogen phosphate, pH 2.4. We used pepsin to digest the protein, which was prepared by diluting pepsin from porcine gastric mucosa with 0.05% trifluoroacetic acid in water in a 1:1 ratio. The pepsin was allowed to dissolve for an hour. 6 µL of pepsin was then added to the protein and allowed to digest for 5 minutes. The sample was then injected into the mass spectrometer and run over a short gradient of 10-45% 0.05% trifluoroacetic acid in acetonitrile for 15 minutes.

Molecular Dynamic Simulations:

The wild-type neuroserpin structure was taken from the PDB (3FGQ). VMD was used to build the original protein file. The protein molecule was then relaxed to find a local minimal energy state, then solvated and ionized to an ionic strength of 50mM of sodium chloride to match the conditions we used experimentally. The protein was then heated to a temperature of 37° C. All simulations and steps were done using the NAMD engine on Case Western Reserve University's high performance computing cluster. The simulations were completed 5 nanoseconds at a time until we reached 50 nanoseconds.

Data Analysis:

Xcalibur was used to view and find peaks in the spectrums generated by the mass spectrometer. Selected peaks were

transferred to MagTran in order to obtain the centroid mass. PyMOL and Chimera were used to create figures of the crystal structure of neuroserpin, and Microsoft Excel was used to map the percentage of deuterium exchanged over time. The percentage of deuterium exchanged was calculated according to the following equation:

$$\%D = \frac{Dt - Do}{Dm - Do} \times 100$$

Where Dt is the mass of a specific peptide after 10, 50, 100, 500, 1000, 2000, or 3000 seconds, Do is the mass of the original peptide before hydrogen/deuterium exchange, and Dm is the mass of the peptide after it has experienced the maximum amount of hydrogen/deuterium exchange. Carma was used to generate correlation and energy diagrams of the different principle components obtained from the molecular dynamic simulations.

RESULTS AND DISCUSSION

Hydrogen/Deuterium Exchange Measured by Mass Spectrometry:

The amide hydrogens of peptides that are exposed to the solvent exchange faster than those that are hidden by the secondary structure of the protein (Table 1). Hydrogen exchange indicates that helices D, F, G and beta sheet A are highly flexible in neuroserpin (Figure 1). Beta sheets A and B are both more labile in neuroserpin than in other serpins such as antitrypsin and antithrombin. Large movements in both these regions are thought to be required during the formation of pathological polymers. The high flexibility seen in neuroserpin may explain why even wild type neuroserpin is more polymerization prone than other serpins. Figure 1 also shows that the reactive center loop (marked in magenta) of neuroserpin exchanges faster in some places and slower in others. The rate of exchange also increases as time progresses, and the protein is allowed to incubate in D2O buffer (Figure 2). Figure 1 also leads us to conclude that different regions of the protein have very different flexibilities due to the highly variant rates of exchange. While some peptides are still exchanging after 1000 seconds, others have finished exchanging after just 10 seconds. The central beta sheets of the wild-type protein each exchange at different rates, which is consistent with its unstable nature. On the other hand, α1-antrypsin is not as wobbly as neuroserpin and experiences a more stable structure, as determined by hydrogen/deuterium exchange and molecular dynamic simulations similar to the ones performed on neuroserpin. Figure 3 also shows that wild-type human neuroserpin is very flexible, since most regions of the protein have large ranges of fluctuation as indicated by the vectors on the protein.
This means that neuroserpin likes to exist as least two stable structures and alpha-1 antrypsin likes to exist in only one stable structure. With this information, we can conclude that neuroserpin is less stable and more flexible than other serpins such as alpha-1 antitrypsin or antithrombin.

Table 1. Select peptides of wild-type human neuroserpin and their hydrogen/deuterium exchange rates at different time points. Peptides in red indicate regions more exposed to solvent.

Peptide	MH	Mass	Deuterated	10s	% exc	50s	% exc	100s	% exc	500s	% exc	1000s	% exc	2000s	% exc	3000s	% exc
A.IADLSVNM.Y	1	862.43	666.5	883.41	0.241	864.4	0.484	864.76	0.572	865.74	0.613	865.78	0.823	866.72	1.054	866.35	0.963
A.VANYINKWVENNTNNL.V	2	953.47	956.66	954.03	0.176	954.34	0.273	956.39	0.915	956.86	1.063	956.83	1.053	955.66	0.687	955.77	0.721
D.ENILFSPLSIA.L	2	602.33	605.22	603.76	0.495	604.12	0.62	603.95	0.561	604.65	0.803	604.71	0.824	605.13	0.969	605.22	1
E.IDLKDVLKA.L	2	507.8	510.77	507.9	0.034	508.03	0.077	510.6	0.943	510.36	0.862	510.49	0.906	510.7	0.976	510.71	0.98
E.IFIKDANL.T	1	933.54	936.04	936.55	0.669	937.33	0.842	937.05	0.76	936.02	0.996	936. 5	1.002	936.12	1.018	936.36	1.071
E.QEIDLKDVL.K	1	1072.6	1075.6	1073.7	0.374	1073.7	0.377	1073.7	0.364	1073.6	0.401	1073.9	0.424	1073.9	0.45	1074.1	0.467
E.VQIPMM.Y	1	718.36	720.91	719.09	0.286	719.08	0.282	719.18	0.322	719.1	0.29	719.2	0.329	719.3	0.369	719.33	0.38
F.HVNEEF.L	1	774.34	780.27	776.18	0.31	776.52	0.368			77 .88	0.428	776.62	0.384	776.78	0.411	776.74	0.405
[illegible]KDANLIGLSDNKEIF.L	2	889.46	894.67	892.98	0.676	893.73	0.82	893.9	0.852	894.13	0.896	894.21	0.912	894.59	0.985	894.49	0.965
F.LEVNEEGSE.A	2	503.21	505.39	503.67	0.211	504.01	0.367	504.66	0.665	503.93	0.33	503.83	0.284	504.36	0.528	504.27	0.486
F.LKEFSNMVT.A	1	1066.5	1074.05	1073.7	0.936	1073.7	0.94	1073.8	0.951	1073.6	0.953	1073.9	0.966	1073.9	0.96	1074.1	1.005
F.LSKAIHKSF.L	1	1030.6	1033.25	1032.6	0.743	1032.7	0.808	1031.8	0.464	1032.2	0.619	1033	0.891	1032.4	0.66	1032.9	0.853
F.MGRVMHPETMNTSGHD.F	2	900.38	904.43	903.05	0.659	903.04	0.657			903.13	0.679	903.1	0.672	903.17	0.669	903.42	0.751
F.TVEQEIDLKDVL.K	2	701.37	704.85	703.88	0.721	704.36	0.859	704.23	0.822	704.81	0.989	704.83	0.994	704.81	0.989	704.81	0.989
F.YYGEFSDGSNEAG GLYQ	1	1728.7	1737.1	1734.1	0.64	1734.4	0.681	1740.9	1.455	1734.7	0.719	1735.1	0.757	1735.8	0.846	1735.5	0.807
L.EVNEEGSEAAAVSGMIA.I	2	832.37	836.7	834.81	0.564	834.84	0.57			834.64	0.524	835.23	0.661	835.4	0.7	835.6	0.746
L.FSPLSIAL.A	2	424.24	427.31	425.14	0.293	425.04	0.261	425.11	0.283	425.22	0.319	425.17	0.303	425.26	0.332	425.47	0.401
L.FVQNGF.H	1	711.34	713.3	712.4	0.541	712.64	0.663			713.07	0.883	713. 2	1.01	713.22	0.959	713.44	1.071
L.SVNMYNRL.R	1	996.49	1002.2	998.39	0.333	997.56	0.187			998.02	0.266	996.67	0.362	999.05	0.448	999.55	0.536
L.VKDLVSPRD.F	1	1028.6	1035.32	1032.6	0.593	1031	0.363	1031.6	0.449	1032.2	0.535	1033	0.649	1032.8	0.625	1032.9	0.636
M.MLVLSRQEVPLATL.E	2	785.45	789.95	786.33	0.196	787.04	0.353	788.7	0.722	788.31	0.636	786.52	0.682	788.62	0.749	788.77	0.738
M.VTAKESQY.V	2	463.23	467.22	464.73	0.376	465.14	0.479	465.29	0.516	465.52	0.574	465.77	0.637	466.14	0.729	466.14	0.729
M.YNRLRATGEDENIL.F	2	832.41	838.78	834.62	0.346	834.84	0.383	834.74	0.367	834.66	0.354	835.22	0.443	835.4	0.471	835.59	0.501
N.LVKDLVSPRDFDAATY.L	2	905.46	912.33	908.12	0.387	908.29	0.412	906.3	0.413	908.46	0.437	908.61	0.459	909.24	0.55	907.91	0.357
Q.VLEIPYEGDEIS.M	1	1363.7	1369.32	1365.6	0.334	1365.8	0.376			1366.5	0.495	1366.7	0.541	1367.6	0.687	1367.3	0.645
S.FLKEFSNM.V	2	508.24	510.77	510.4	0.854	510.4	0.854	510.56	0.917	510.54	0.909	510.49	0.669	510.7	0.972	510.72	0.96
S.FTKDDESEVQIPMM.Y	2	835.37	838.76	837.42	0.605	837.35	0.584	836.89	0.448	837.45	0.614	837.49	0.625	837.68	0.681	837.79	0.714

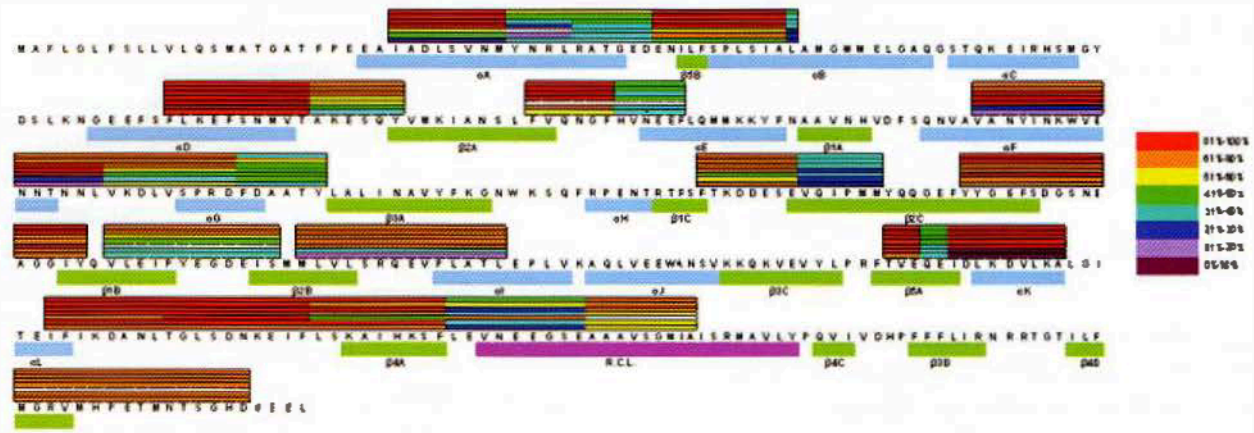

Figure 1. Rate of hydrogen/deuterium exchange of specific peptides at different time points (10, 50, 100, 500, 1000, 2000, and 3000 seconds) with 10 seconds being closest to the sequence and 3000 seconds being farthest away. Alpha helix regions are marked in blue, beta-pleated sheet regions are marked in green, and the reactive center loop is marked in magenta.

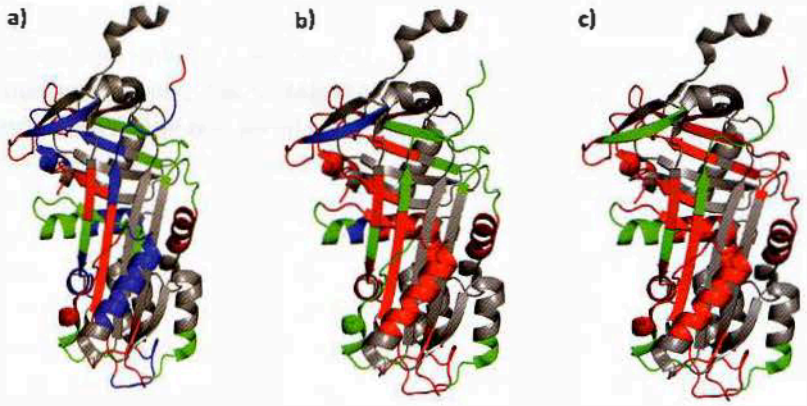

Figure 2. Rate of hydrogen/deuterium exchange of certain peptides at different time points where a) is after 10 seconds of exchange, b) is after 500 seconds of exchange, and c) is after 3000 seconds of exchange. Blue symbolizes 0%-30% exchange, green symbolizes 31%-60% exchange, and red symbolizes 61%-100% exchange.

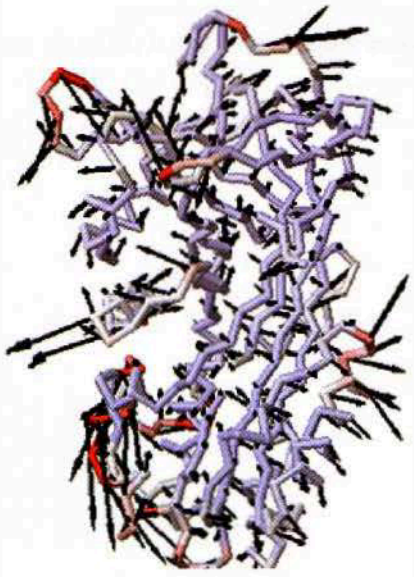

Figure 3. Vector diagram of wild-type human neuroserpin. Longer vectors equate to greater fluctuations and the red regions are more mobile than the blue regions.

Figure 4. Fluctuations of the different regions of wild-type human neuroserpin, where more compact and solid regions equate to regions of less flexibility and regions that occupy a larger range of space indicate peptides that display more mobility.

Molecular Dynamic Simulations:

The protein on a global level is very mobile and unstable, as shown by Figures 3 and 4. The more flexible regions allow for easier exchange between deuterium and the amide hydrogens. Molecular dynamics simulations of both the wild-type and a His338-->Arg mutant structure were generated using VMD (Figure 6). The correlation diagrams (Figure 5) show that most of the alpha helical structures move in the same directions (shown in red) whereas other regions of the protein, such as beta sheets, either move in opposite directions (shown in blue) or independently of one another (shown in yellow). As we can see, most of the red that goes down the diagonal line from top left to bottom right corresponds to the movement of the alpha helices. When we compare the wild-type (Figure 5 left) to the mutant (Figure 5 right), we can see that the His338-->Arg mutation alters the global dynamics of neuroserpin, leading to more correlated and anti-correlated motions (Figure 5). Locally, the mutants show distorting motions at the top of beta sheet A (Figure 6 right) and increased mobility in helix F (Figure 6 left). Disruption of both sheet A and helix F has been linked to polymer formation in other serpins. The perturbed structure and dynamics in these regions in the pathological His338Arg mutant of neuroserpin may facilitate polymer formation. The free energy landscape diagram of the principle component planes shows that wild-type human neuroserpin has at least two well-populated energy wells (Figure 7 left), whereas alpha-1 antitrypsin only has one well-populated energy well (Figure 7 right).

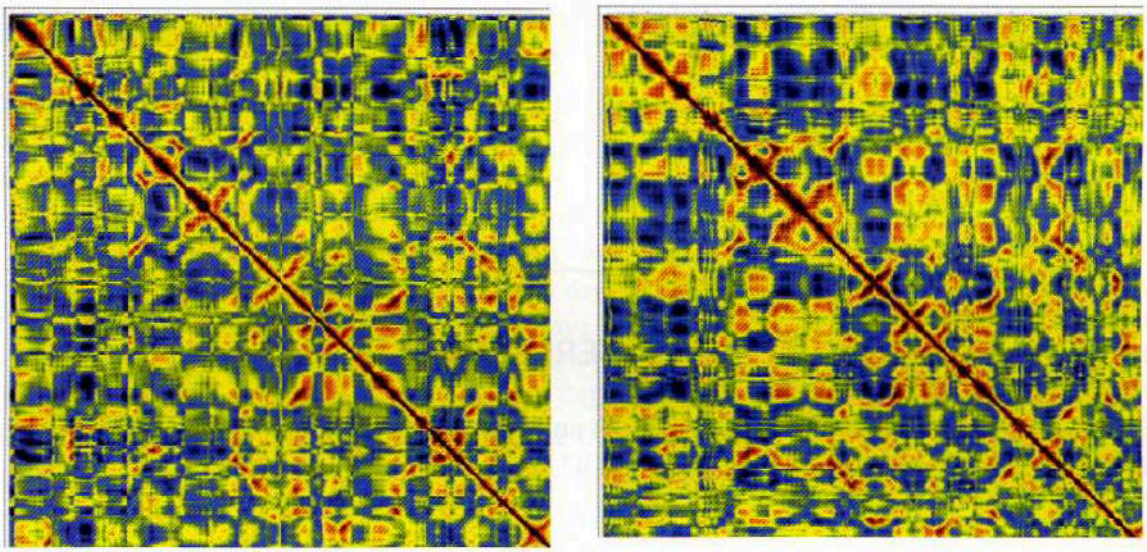

Figure 5. Variance-covariance matrices of wild-type human neuroserpin (left) and neuroserpin mutant (right). Red regions indicate residues that move in a correlated manner, blue regions indicate residues that move in an anti-correlated manner, and yellow regions indicate residues that move independently.

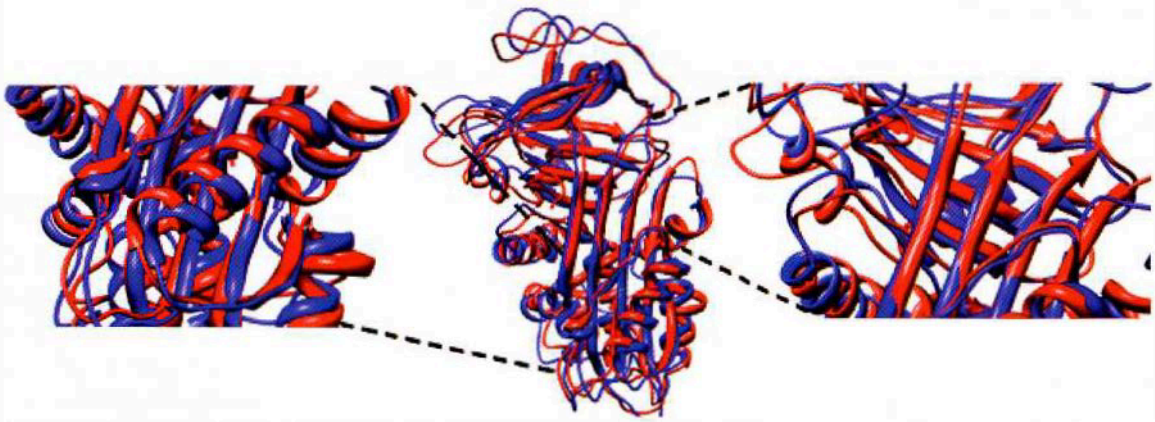

Figure 6. Molecular dynamic simulations of wild-type neuroserpin, in red, superimposed upon its mutant structure, in blue. Distinguishing factors between the two structures are emphasized by focusing on the f-helix (left) and the central beta-sheet (right).

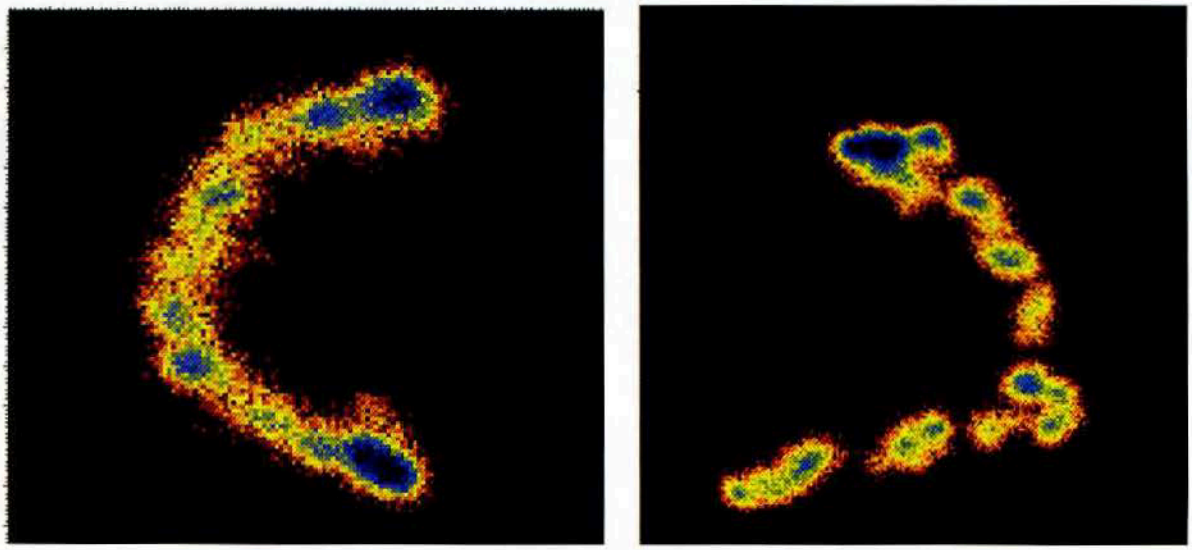

Figure 7. Free energy landscape diagram on the principle component planes defined by the eigenvectors corresponding to the first and second highest eigen values. The one on the left is of wild-type human neuroserpin and the one on the right is of α1-antitrypsin.

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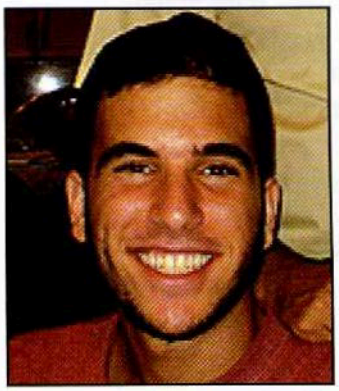
ATF3 INDUCTION IN AXOTOMIZED MOUSE SYMPATHETIC PREGANGLIONIC, SENSORY, AND MOTOR NEURONS

ABSTRACT

Depression can significantly impair overall functioning and the global prevaActivating transcription factor 3 (ATF3) is undetectable in intact rat sympathetic, sensory, and motor neurons, but is up-regulated in these neurons after axonal injury. Consequently, ATF3 has been proposed as a novel neuronal marker of nerve injury. The main purpose of this study was to examine ATF3 in the mouse spinal cord after transection of the preganglionic cervical sympathetic trunk (CST) and the sciatic nerve. The retrograde tracer fluorogold was applied directly to the transected CST and used to determine the location of neurons in the spinal cord that project to the superior cervical ganglion (SCG). ATF3-like immunoreactivity (ATF3-IR) was sensitive enough to detect elevated levels of ATF3 in the spinal cord 5 days after CST transection. The labeling was sustained at 7, 14, and 21 days. Fluorogold labeled neurons in the intermediolateral nucleus (IML) of the T1 spinal cord segment overlapped one to one with neurons expressing ATF3-IR, indicating that these were preganglionic sympathetic neurons. Since the CST has both afferent and a small number of efferent axons, ATF3-IR was also examined in the superior cervical ganglia (SCG). A few labeled neurons and many labeled non-neuronal cells were found in the SCG 7 days after decentralization. After transection of the sciatic nerve, ATF3-IR was detected in sensory neurons in the L4 – L5 dorsal root ganglia (DRG) 7 days after the lesion and in ventral motor neurons of the L3 spinal cord segment 3 days after the lesion. Although in some central neural systems up-regulation of ATF3 is only detectable at a distance of 500μm between the lesion and neuronal cell body, the data here indicates that ATF3 up-regulation in the peripheral nervous system is possible at considerably larger distances.

INTRODUCTION

After injury to the spinal cord or brain, neurons usually do not regenerate. This lack of regeneration is a major reason why injuries in the central nervous system are permanent. This is not the case for injuries in the peripheral nervous system. Injury to a peripheral nerve triggers the formation of new membranes, assembling of cytoskeletal proteins, and expression of multiple adhesion molecules (Sun and He, 2010). These prerequisites for regeneration are not found after most axonal damage within the central nervous sys-



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-Acknowledgments-

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tem. Successful peripheral nerve regeneration is thought to depend on the up-regulation of a number of genes in the neuronal cell body. Some of these gene products are themselves transcription factors. Activating transcription factor 3 (ATF3) is one of these up-regulated gene products and has been proposed as a novel neuronal marker of peripheral nerve injury (Tsujino et al., 2000).

ATF3 plays a key role in nerve regeneration. Seijffers et al. (2007) found that transgenic mice who constitutively express ATF3 exhibit almost double the nerve regrowth after injury to motor and sensory neurons. In the sympathetic nervous system, ATF3-like immunoreactivity (ATF3-IR) has been detected in rats as early as 6 hours and as late as three weeks after axotomy while uninjured rats showed almost no ATF3-IR (Sachs et al., 2007). Tsujino et al. originally proposed ATF3 as a marker of nerve injury in the rat after discovering ATF3 in the dorsal root ganglia (DRG) and in the ventral horn of the spinal cord following axotomy of the sciatic nerve. Again, there was no ATF3 detected in the uninjured DRG or uninjured spinal cord.

The activity of this transcription factor is not limited to the nervous system. ATF3 induction had been detected in a variety of tissues under stress: the heart after myocardial ischemia, the liver after hepatic ischemia, and the skin after wounding. ATF3 is considered a member of the ATF/cyclic AMP responsive element binding family of transcription factors and is defined by a basic region-leucine zipper domain. As a homodimer ATF3 is a transcriptional repressor, but its activation state is altered when it forms heterodimeric complexes with key regeneration proteins like c-Jun, JunD, JunB, ATF2, or gadd153/CHOP10 (Chen et al., 1996 and Hai and Hartman, 2001). While most studies detect ATF3 in the nucleus of neurons and/or glial cells, Lindwall and Kanje (2005) suggested the transcription factor also has a functional role outside the nucleus as a signaling molecule that is retrogradely transported from the site of axonal injury to the nucleus.

In the present study, we sought to examine ATF3-IR as a marker of preganglionic sympathetic, sensory, and motor neuron injury in the mouse spinal cord and ganglia. While there have been studies of rat preganglionic neurons using retrograde tracers (Rando et al., 1981; Anderson et al., 1989; Baldwin et al., 1991; Zigmond et al., 2007; Sachs et al, 2007; Huang et al., 2010), there have been no studies investigating ATF3 in preganglionic neurons. There have also been no studies examining ATF3 induction in the mouse spinal cord after a sciatic nerve transection. Rigaud et al. (2008) elucidated significant differences in the rostrocaudal distribution of sensory nerves contributing to the sciatic between rats and mice, and even among different strains of mice. These differences have not yet been explored in mouse preganglionic neurons.

The cervical sympathetic trunk (CST) is largely preganglionic with many axons whose cell bodies are located in the spinal cord and whose terminals are located in the superior cervical ganglia (SCG) (Bowers and Zigmond, 1979; Rando et al., 1981). In the present study, the CST was cut near the SCG, a preganglionic transection also referred to as decentralization. However, the CST does have both afferent and efferent axons, so ATF3-IR was examined in the SCG. The preganglionic neurons in the spinal cord were identified with the retrograde tracer fluorogold. Fluorogold transport and ATF3-IR were also examined after sciatic nerve axotomy in the spinal cord and DRG.

MATERIALS AND METHODS

Surgeries

Male C57BL6/J mice (20 – 35 g, Jackson Laboratories) were anesthetized with 20% ketamine/10% xylazine in 0.9% NaCl intraperitoneally. A female mouse was used for the 14 day SCG decentralization. The CST was cut unilaterally several millimeters caudal to the SCG and about 1.3 cm from the spinal cord, and the proximal segment of the CST was then placed in a well formed by cutting the bottom of an Eppendorf tube. For the mice sacrificed after 5 and 7 days, 4 – 5 µL of 4% fluorogold (Fluorochrome, Inc., Denver, CO) was added to the well. For the mice sacrificed after 14 and 21 days, 5% fluorogold was applied. After the CST was secured in the fluorogold solution for 30 min, the CST was removed from the well, and the incision was closed with wound clips. The four mice were perfused either 5, 7, 14, or 21 days after surgery with 0.9% NaCl followed with a 16% paraformaldehyde solution.

The spinal cord from C8 to T5 was removed in each mouse that underwent an SCG decentralization. The spinal cord segments C8 and T5 were determined by identifying the last rib, which is aligned with T12. Spinal cord segments were defined by the corresponding vertebrae. Each individual spinal cord segment was later distinguished by assuming each segment was the same length. Both the

decentralized and contralateral (control) SCGs were removed for the mice at survival times of 7,14, and 21 days except in the case of the 14-day survival where the control SCG was unrecoverable. For a comparison to the SCG decentralization, a separate mouse was subjected to an SCG axotomy, where the internal and external carotid nerves were transected. The axotomized and contralateral SCG were the only tissue removed in this mouse.

The mouse with a 14-day survival time was the same mouse used for the sciatic nerve axotomy. Three days before the animal was sacrificed, the left sciatic nerve was axotomized. The sciatic nerve axotomy was approximately 1.6 cm from the L5 spinal cord segment and 2.2 cm from the L3 spinal cord segment. DRG were less than 1 mm from their respective spinal cord segment. Along with the removal of the C8 – T5 spinal cord segments and SCG, the L3 – L5 spinal cord segments, the L3 – L5 DRG on both sides of the mouse, and the sciatic nerves on both sides of the mouse were removed. A separate mouse had the same sciatic nerve procedure, but was sacrificed after 7 days.

All tissue was immersion fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), cryoprotected with 30% sucrose, and stored overnight at 4°C. Tissue was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Inc., USA) and sectioned in a cryostat at 10μm at -20°C.

Immunohistochemistry

Sections were treated with 5% normal donkey serum in PBS/Triton X-100 for 1 hour, and then incubated with rabbit anti-ATF3 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. The sections were then washed three times with PBS and incubated for 45 min with CY3-donkey anti-rabbit IgG F(ab')2 (1:400; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). All incubations were at room temperature.

The fluorogold labeled and ATF3-IR sections were viewed using a Leitz Diaplan 512 Microscope. When imaging for fluorogold, sections were examined with a UV filter (Leitz excitation filter block A; no. 513596). When imaging for ATF3-IR, sections were viewed with a CY3 filter (Leica N2.1). The imaging software was Simple PCI (Hamamatsu), and images were set to the same exposure and gain. Images were photographed at 25X (unless otherwise noted), and all images were adjusted to identical brightness and contrast settings.

RESULTS

Fluorogold labeling and ATF3-IR overlap in T1 spinal cord segment

Five days after CST transection, neurons within the intermediolateral nucleus (IML) in the T1 spinal cord segment showed fluorogold labeling (Figure 1A). There was no labeling outside the IML, and there was also no fluorogold labeling in the T2 or T3 spinal cord segments (not shown). The same spinal cord cross-sections were stained for ATF3 with a CY3 conjugated secondary antibody (Figure 1B). Every neuron that was labeled with fluorogold expressed ATF3-IR as indicated by the arrows in Figures 1A and 1B. The uninjured contralateral side of the spinal cord sections did not display any fluorogold labeling or ATF3-IR. Similarly, mice with survival times of 7 and 14 days also showed overlapping fluorogold labeling and ATF3-IR only in the T1 spinal cord segment (not shown).

Using the retrograde tracer horseradish peroxidase (HRP) in the rat, Rando et al. (1981) and Anderson et al. (1989) both detected neurons that project to the SCG from spinal cord segments C8 to T5 with the highest levels of staining distributed in the IML of spinal cord segments T1, T2, and T3. The survival times used in those studies ranged from 1 to 9 days. In the present study, since after 5, 7, and 14 days there was only fluorescence in the T1 spinal cord segment, one mouse was sacrificed 21 days after injury (not shown). In this mouse, the only clear staining for ATF3-IR was in the T1 spinal cord segment. There was only faint fluorogold detectable in T1, and there were no labeled or stained cells in C8. Faint fluorogold labeling and ATF3-IR was detected in a rostral segment of T2.

ATF3-IR in SCG 7 Days after decentralization, but not after 14 and 21 days.

Seven days after SCG axotomy or decentralization, the SCG contained ATF3-IR in neurons and nonneuronal cells (Figure 2C). The axotomized SCG exhibited mostly neuronal ATF3-IR (Figure 2B). Neuronal ATF3-IR was differentiated from staining of satellite cells by the larger nucleus and the greater amount of cytoplasm surrounding the nuclei of neurons. Overall, the axotomized SCG de-

Figure 1. Fluorogold (A) and ATF3-IR (B) in the IML of the T1 spinal cord segment 5 days after SCG decentralization. Immediately following decentralization the CST was exposed to 4% fluorogold. A. Fluorogold detected under UV light at 25X. B. ATF3-IR under the CY3 filter at 25X. Every neuron with fluorogold also stained for ATF3 as indicated by the arrows. C. The spinal cord segment under white light at 6.3X. The ventral side is the lower half of the image. The box indicates location of the labeled cells.

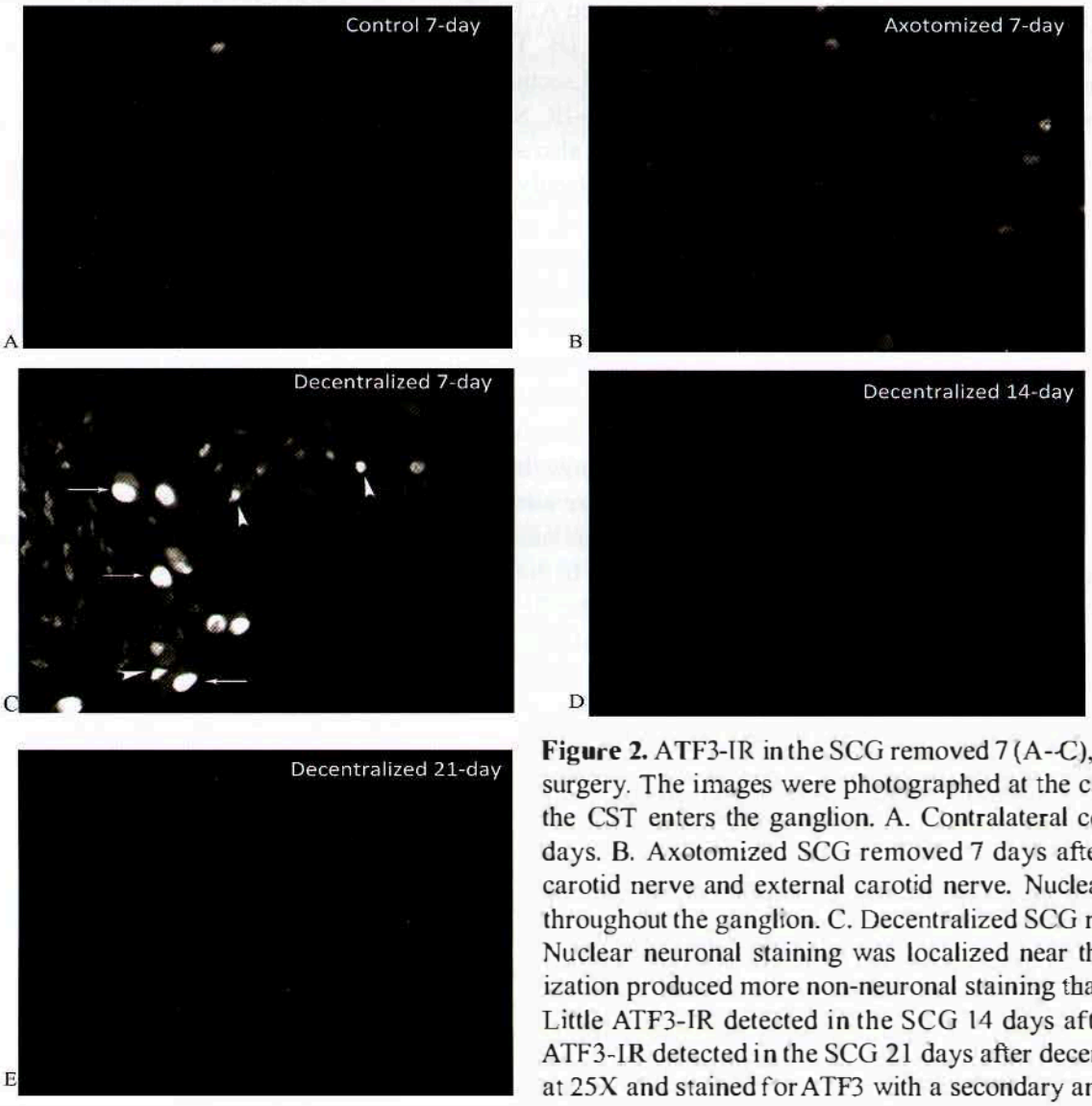
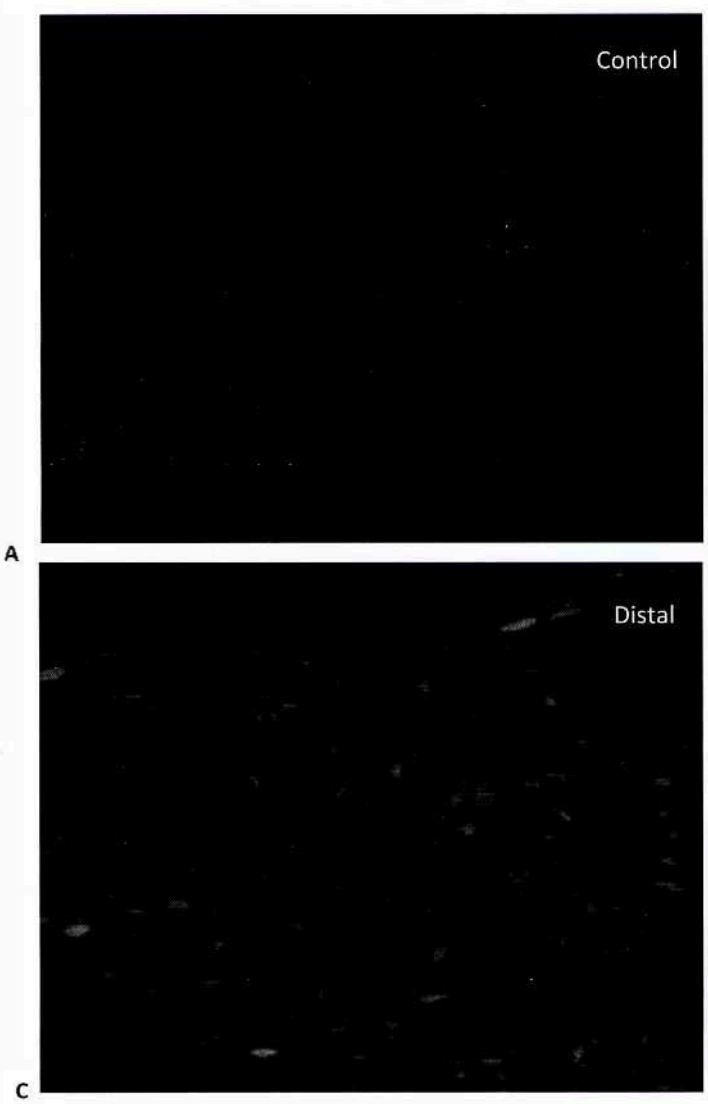


Figure 2. ATF3-IR in the SCG removed 7 (A--C), 14 (D), and 21 days (E) after surgery. The images were photographed at the caudal end of the SCG where the CST enters the ganglion. A. Contralateral control SCG removed after 7 days. B. Axotomized SCG removed 7 days after transection of the internal carotid nerve and external carotid nerve. Nuclear staining was predominant throughout the ganglion. C. Decentralized SCG removed 7 days after surgery. Nuclear neuronal staining was localized near the CST (arrows). Decentralization produced more non-neuronal staining than axotomy (arrowheads). D. Little ATF3-IR detected in the SCG 14 days after decentralization. E. Little ATF3-IR detected in the SCG 21 days after decentralization. All images were at 25X and stained for ATF3 with a secondary antibody labeled with CY3.



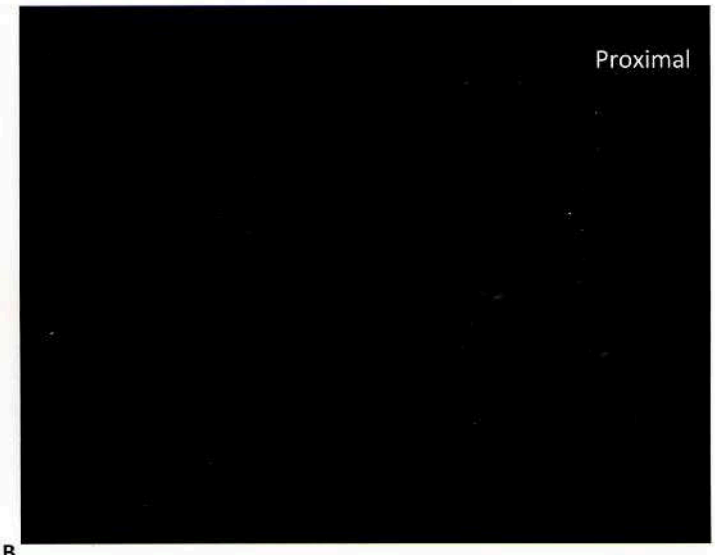


Figure 3. ATF3 in the proximal (B) and distal (C) segments of sciatic nerve 3 days after axotomy. A. Sciatic nerve at a mid-thigh level contralateral to axotomy site. This nerve was not subjected to any injury. B. Proximal side of the sciatic nerve transection removed 3 days after injury. The image was taken near the transection site on the proximal segment. The opposite end of the proximal segment had no staining (not shown). C. Distal side of the sciatic nerve transection removed 3 days after injury. The image was taken near the transection site on the distal segment. The opposite end of the tissue demonstrated moderate levels of ATF3-IR (not shown) and was noticeably less than ATF3-IR at the transection site. The combined length of the proximal and distal sections removed

picted a wider distribution of moderate staining while the decentralized SCG demonstrated more prominent staining in the caudal end of the SCG where the CST enters the ganglion. There was little ATF3-IR in the SCG 14 and 21 days after decentralization (Figure 2D and 2E).

ATF3-IR in the sciatic nerve 3 days after axotomy

The highest concentration of ATF3-IR in an axotomized sciatic nerve was on the distal segment of the nerve near the site of transection (Figure 3C). Moderate ATF3-IR was detected throughout the remaining distal segment (not shown), and light staining was detected on the proximal segment near the site of transection (Figure 3B). There was little staining in the remaining portion of the proximal segment (not shown), and there was no detectable ATF3-IR in the uninjured sciatic nerve (Figure 3A).

ATF3 concentrated in L5 DRG 3 and 7 days after sciatic nerve axotomy

There were little or no detectable levels of ATF3-IR in the L3 or L4 DRG 3 days after sciatic nerve axotomy (Figure 4B and 4D). The L5 DRG demonstrated strong ATF3-IR after 3 days (Figure 4F). The staining in the L5 DRG was less bright and less concentrated in another mouse sacrificed 7 days after axotomy (Figure 5C). Rigaud et al. (2008) reported the highest level of staining of the retrograde tracer True Blue in the L3 and L4 mouse DRG 5-6 days after the sciatic nerve was injected with the tracer. In the study presented here, it was only 7 days after injury that ATF3-IR was detected in the L4 DRG (Figure 5B), but even after 7 days there was no ATF3-IR in the L3 DRG (Figure 5A).

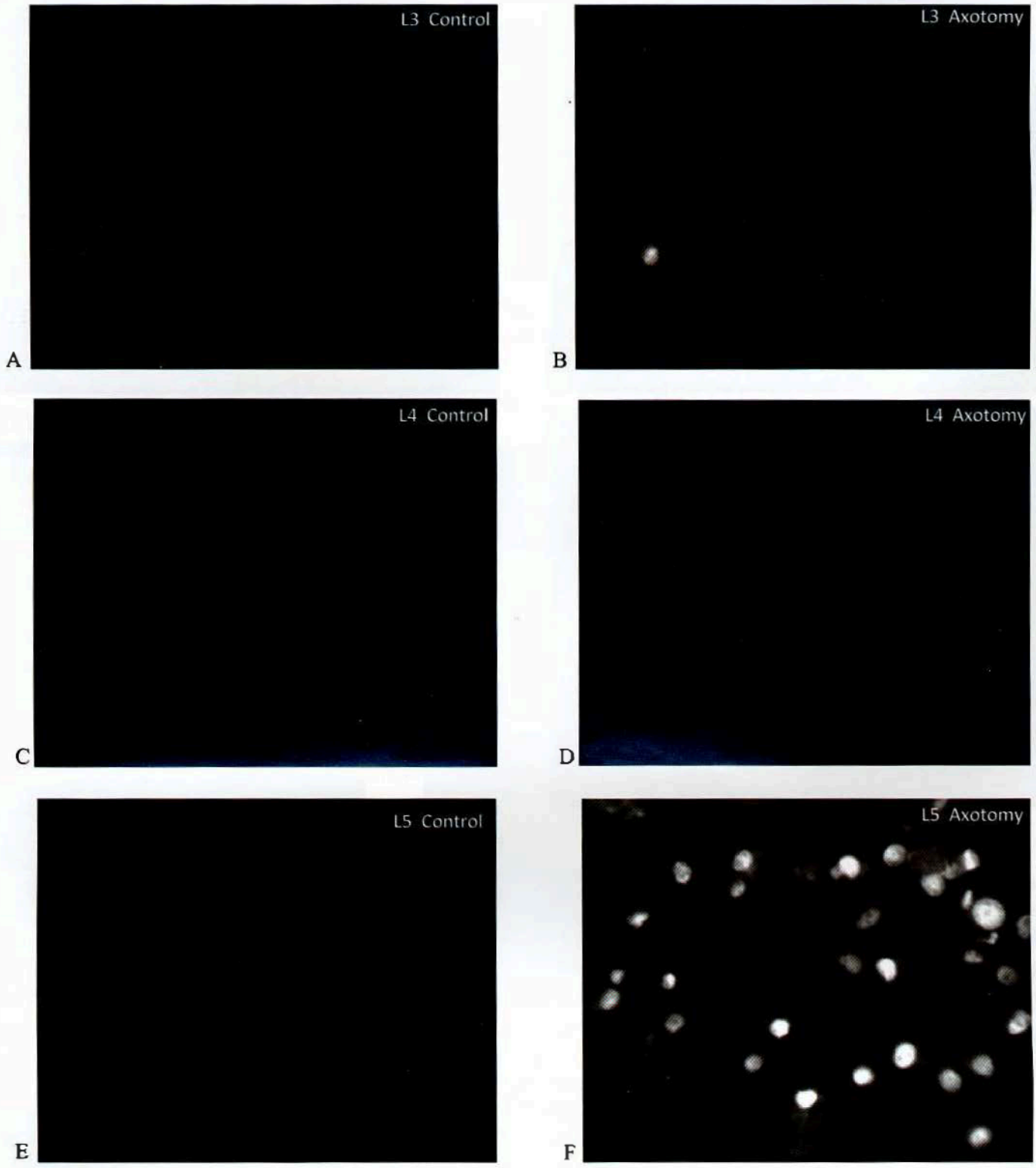


Figure 4. ATF3 in L3 (A and B), L4 (C and D), and L5 (E and F) DRG removed 3 days after sciatic nerve axotomy. A. Control L3 DRG. B. L3 DRG ipsilateral to sciatic nerve transection. C. Control L4 DRG. D. L4 DRG ipsilateral to sciatic nerve transection. E. Control L5 DRG. F. L5 DRG ipsilateral to sciatic nerve transection. All images were at 25X and stained for ATF3 with a CY3 labeled secondary antibody.

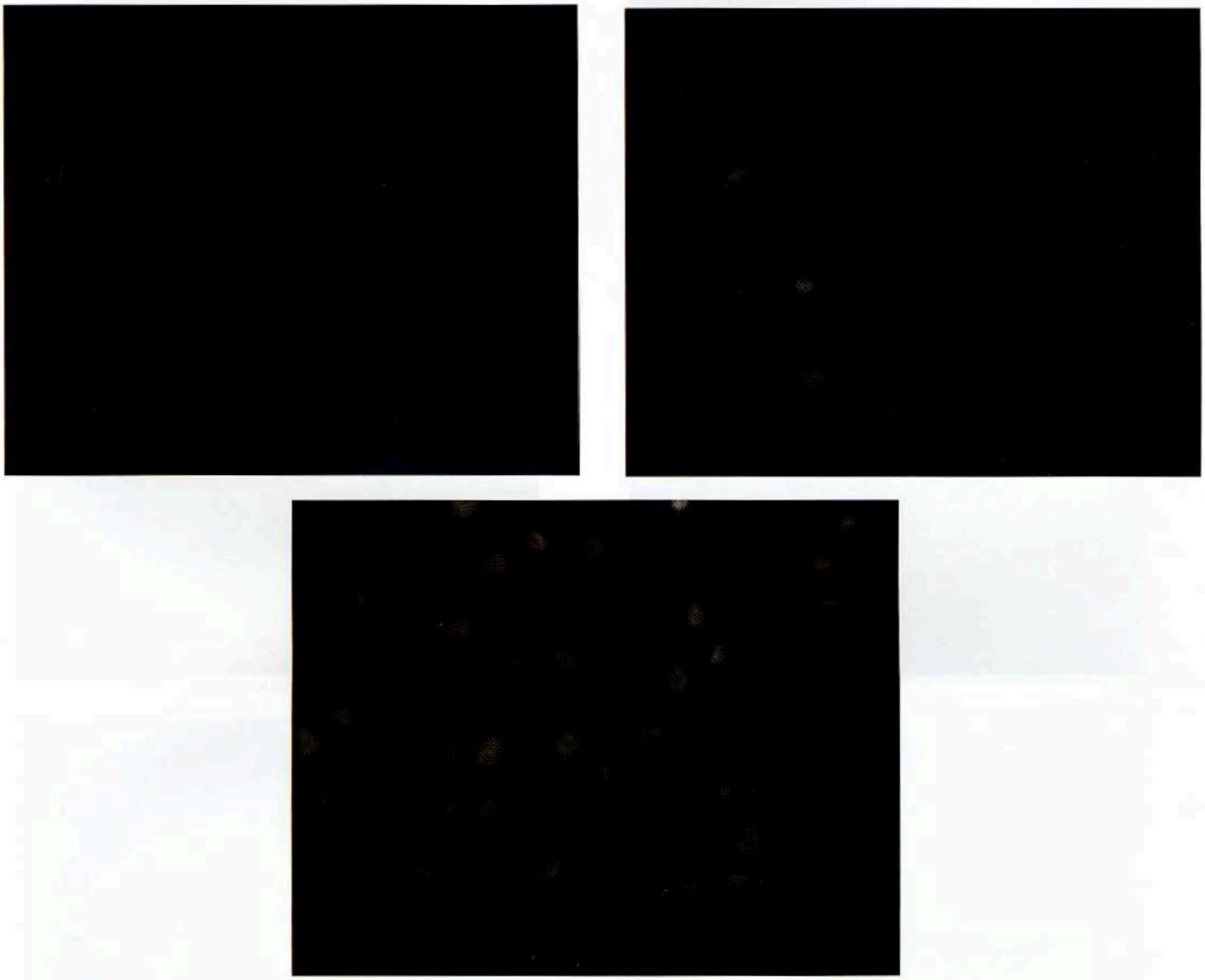


Figure 5. ATF3 in L3 (A), L4 (B), and L5 (C) DRG 7 days after sciatic nerve transection. The controls on the contralateral side of the same animal showed little or no staining (not shown). All images were at 25X and stained for ATF3 with a secondary antibody conjugated with CY3.

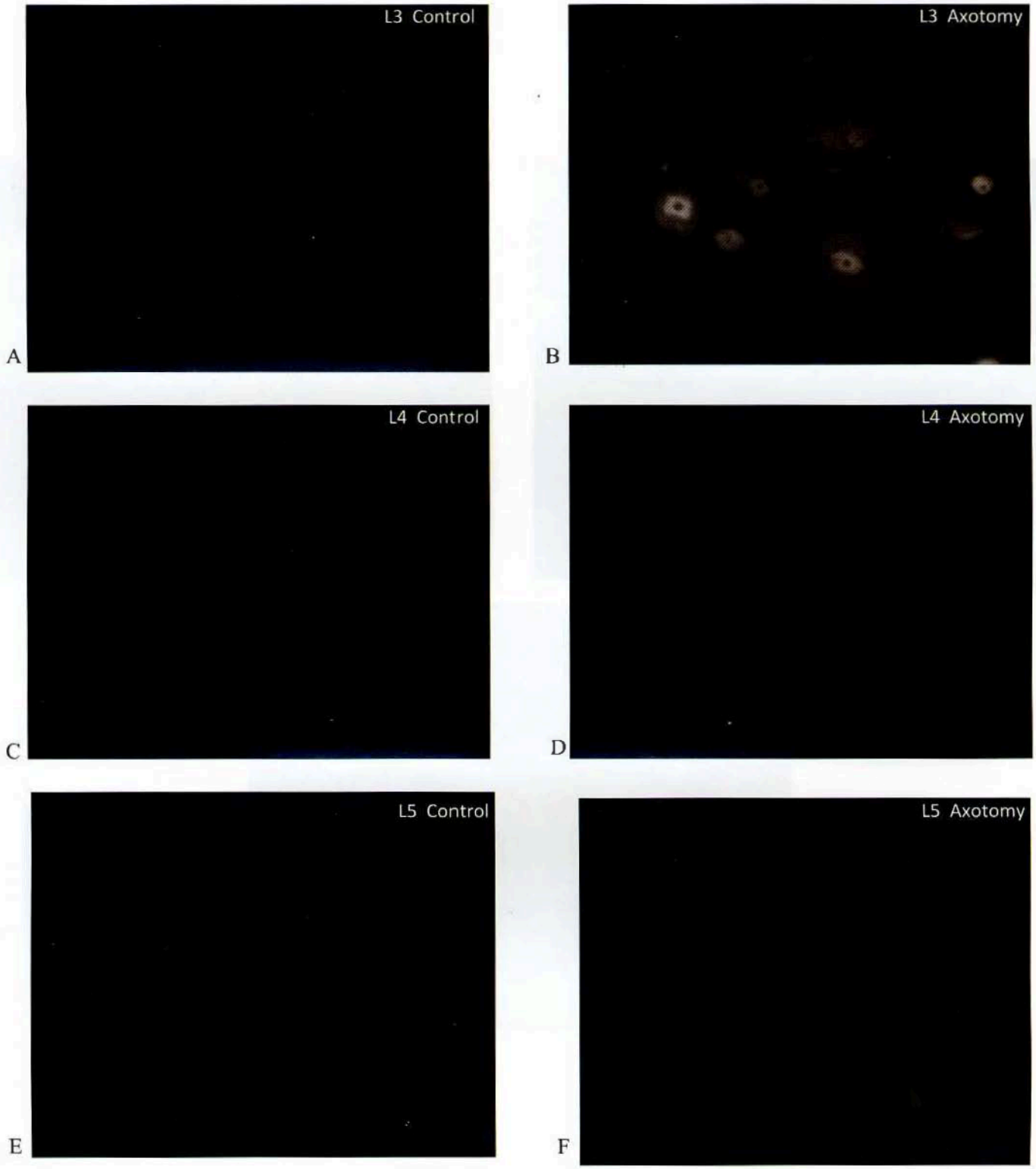


Figure 6. ATF3 in the ventral horn of the L3 (A and B), L4 (C and D), and L5 (E and F) spinal cord segments. The mouse was sacrificed 3 days after a sciatic nerve transection. The images in the left column (A, C, E) were photographed in the ventral horn on the uninjured side of the mouse. The images in the right column (B, D, E) were taken in the ventral horn on the axotomized side of the mouse. All images were at 25X and stained for ATF3 with a secondary antibody conjugated with CY3.

ATF3 only detectable in L3 spinal cord segment 3 days after sciatic nerve cut

Unexpectedly, only the ventral horn in the L3 spinal cord was positive for ATF3-IR. The size of the neurons was indicative of motor neuron staining. The results were surprising because ATF3-IR was only detected in the L4 and L5 DRG (Figure 4F, 5B, 5C).

DISCUSSION

In the present study, ATF3-IR was a successful marker of axotomized sympathetic preganglionic neurons in the mouse spinal cord. The unilateral application of the retrograde tracer fluorogold to the cut CST of the mouse resulted in labeling of neurons almost always in the T1 spinal cord segment (Figure 1). The 7 – 10 neurons labeled in each section were confined to the IML. Every neuron labeled with fluorogold also contained ATF3-IR and vice versa. There was no fluorogold labeling or ATF3-IR contralateral to the side of injury.

Previous research in rats with the retrograde tracer horseradish peroxidase (HRP) has also illustrated that preganglionic neurons are largely contained in the IML. Researchers investigating the sympathetic neurons in the rat have also reported a smaller number of preganglionic neurons in regions neighboring the IML: the intercalated nucleus and central autonomic region of the grey matter and the lateral funiculus in the white matter. Furthermore, these studies in the rat find the largest number of stained somata in the T1, T2, and T3 spinal cord segments with T2 having slightly more stained neurons (Rando et al., 1981; Anderson et al., 1989).

The present study was the first time fluorogold was applied to the transected CST in the mouse. The data here suggests there is a difference in the rostrocaudal and lateral distribution of preganglionic neurons in the mouse compared with earlier studies in the rat. To confirm that the complete rostrocaudal extent of preganglionic neurons was detected, the mice in the present study were examined at four different survival times. After 21 days, there was faint fluorogold labeling andATF3-IR in a rostral section of the T2 spinal cord segment. The staining was much stronger and more concentrated in the T1 spinal cord segment.

A cautionary note needs to be explained when comparing the spinal cord results of different studies: investigators demarcate spinal cord segments in different ways. Rando et al. defined each segment by the midpoint between the adjacent ventral roots while Anderson et al. delineated each segment by the point of entry of each root. The present study approximated the spinal cord segment by removing a section of the spinal cord and estimating each segment to be of equal length. Even with these differences in defining spinal cord segments, it is worth referencing these other studies because we detected ATF3-IR and fluorogold largely in only one spinal cord segment. The data here suggests the mouse neurons that project to the SCG through the CST may be more concentrated in one spinal cord segment than the same preganglionic neurons in rats. Examining different tissues over three weeks, provided insight into the way different nervous tissues up-regulate ATF3. ATF3-IR was detected in the spinal cord at every time point examined. Elevated levels of ATF3 were sustained for at least 21 days while the fluorogold fluorescence began to fade by 21 days. The prolonged induction of ATF3 in the spinal cord after transection is a result that serves as a counterexample to the models described by Hai and Hartman (2001) where ATF3 induction was transient. ATF3-IR was not sustained for more than 14 days after the SCG was decentralized. This contrasts with what others have found after SCG axotomy in the rat, which involves transecting postganglionic instead of preganglionic neurons. Sachs et al., 2007 reported elevated levels of ATF-IR in the SCG 21 days after axotomy with significant Schwann cell staining after 48 hours. This experiment provides evidence suggesting that preganglionic neurons up-regulate ATF3 in different ways from postganglionic neurons after injury.

While the tissue of the mice with a sciatic nerve injury were only examined at one time interval, the data provides a window into the complexity of sciatic nerve innervation. The L4 and L5 DRG were positive for ATF3-IR (Figure 3), but ATF3-IR was only detected in motor neurons of the L3 spinal cord segment (Figure 4). Rigaud et al. (2008) reported the highest level of staining of the retrograde tracer True Blue in the L3 and L4 mouse DRG 5-6 days after injury, and did not explore the spinal cord. The present study raises interesting questions about the way cell bodies of DRG neurons project their central axons.

In both types of injures performed in this experiment, SCG decentralization and sciatic nerve axotomy, ATF3-IR was detected in the nuclei of the neurons at a considerable distance from the site of injury. This is significant because Mason et al. (2003) reported that corticospinal neurons in the central nervous system were only capable of expressing growth-associated genes following a very proximal lesion. Specifically, ATF3 was not detectable in the corticospinal tract when the lesion was more than 0.03 - 0.05 cm away from the cell body. In this experiment, the CST transection was about 1.3 cm from the spinal cord, and the sciatic nerve axotomy was 1.6 cm from the L5 DRG and 2.2 cm from the L3 DRG. Based on the positive ATF3-IR in the spinal cord after SCG decentralization and sciatic nerve axotomy, the present study indicates that preganglionic, sensory, and motor neurons are not sensitive to the distance between the cell body and lesion. The ability for peripheral nerves to regenerate after a long-distance transection may be one reason why neurons usually regenerate after injury in the peripheral, but not central nervous system.

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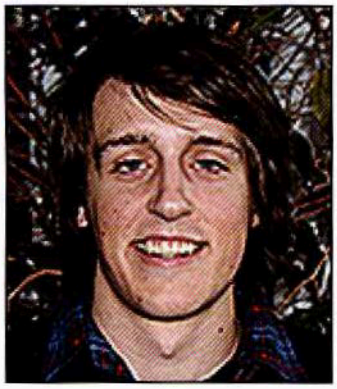
The Limits of Neuroaesthetics



Benjamin Kubit

Ben Kubit is currently a forth year student at Case Western Reserve University majoring in both Psychology and Cognitive Science along with minors in Philosophy and Music. He is currently a lab assistant in the Brain, Mind and Consciousness lab under the direction of Tony Jack. His research is based on current neuroscience literature proposing a similar neural basis for attention reorientation and aspects of theory of mind. After graduation, he plans on attending graduate school in the field of cognitive neuroscience.

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INTRODUCTION

Traditionally, the philosophical field of aesthetics has concerned itself with the nature of art and the creation and appreciation of beauty. Despite the field being around for several centuries, there still remains debate over how to define the term art. Through the years philosophers have provided many different definitions. Aristotle understood art as a kind of mimesis, or imitation of natural objects (Aristotle, 1995). According to Aristotle, the ultimate purpose of art is the catharsis of emotions, and by catharsis he means the power a work of art has to arouse emotions. Different kinds of art, such as music and poetry, differ in the medium of mimesis, objects of the mimesis and the mode of mimesis. Importantly, Aristotle believed that humans experience pleasure from this mimesis because "great pleasure is derived from exercising the understanding" (p. 490). He and many other early philosophers believed that the aesthetic experience is inherent in the aesthetic object or event, rather than something that is the result of the human mind. The philosopher Immanuel Kant defines art as representation "that is purposive in itself and, though without an end, nevertheless promotes the cultivation of the mental powers for sociable communication" (Adajian, Traditional Definitions ¶ 2). He claims that beautiful art evokes aesthetic ideas that encourage much thought, but that these aesthetic ideas are fundamentally different from rational ideas. More recently, philosophers have focused less on the objects and purpose and more on the cognitive nature of art. The recent philosopher, Monroe Beardsley offers a more contemporary definition:

> [Art is] either an arrangement of conditions intended to be capable of affording an experience with marked aesthetic character or (incidentally) an arrangement belonging to a class or type of arrangements that is typically intended to have this capacity (p. 299).

He claims that art doesn't have to be created with the intention of producing an aesthetic experience for viewers. An object can have utilitarian function and still be considered art as long as it is capable of providing an experience with "marked aesthetic character".

Neuroaesthetics is a much more recently developed field of study than traditional philosophical aesthetics. The field has its roots in the experimental aesthetic research performed by Berlyne in the 1960s and 1970s. Berlyne collected behavioral data regarding the different effects certain qualities

of paintings and music have on a person's physiological arousal (Berlyne, 1974). By identifying and measuring stimulus qualities such as complexity, ambiguity, surprise, novelty and incongruity, Berlyne helped create the possibility of investigating the neural properties responsible for such characteristics of art. Before the widespread availability and use of imaging techniques, Arnheim also attempted to "apply approaches and findings of modern psychology to the study of art" (Arnheim, p. vii). His experiments involving the visual perception of art are greatly influenced by gestalt theory and, like Berlyne, attempt to provide an understanding of the appreciation of art that is grounded in psychology, rather than philosophy.

Developments in the methodology of neuroscience research has allowed for the in vivo imaging of the human brain. Researchers interested in understanding aesthetic experiences in terms of the neural networks responsible can now utilize brain imaging tools to test hypotheses. However, neuroaesthetics is not confined to strictly brain imaging, but any approach to gain knowledge on the neural mechanisms involved in aesthetics both experimental and theoretical. In his essay entitled Artistic Creativity and the Brain (2001), Semir Zeki describes the goal of the field as the study of "the neural basis of artistic creativity and achievement" (p. 52). In several different articles, Zeki has attempted to explain the visual perception of art in terms of the response characteristics of neurons in the visual cortex.

Recently, the field of neuroaesthetics has received more and more attention from researchers around the world. Papers have been published attempting to shed light on what constitutes the neural correlates of beauty (Kawabata & Zeki, 2004) or the neural substrates of spontaneous musical performance (Limb & Braun, 2008) and while the results have been informative on several levels, they unfortunately tend to suggest interesting hypotheses but none-the-less remain inconclusive. Although researchers have been able to observe the activations of various areas to music and paintings, what the activation patterns mean and under what conditions they were actually evoked is not clear. Brain imaging experiments have demonstrated the large difference between recording the blood oxygen level-dependent (BOLD) responses and understanding what these responses actually mean. Despite these difficulties, the ability to end centuries of aesthetic intrigue and discussion by presenting concrete neurological explanations for aesthetic events and objects is enticing. However, before the results of neuroaesthetic experiments are interpreted and new experiments are planned, it is necessary that the limits of such studies be recognized. In this paper I will attempt to identify the restrictions and requirements of a neuroscience approach to the study of aesthetics. I will begin by establishing the evolutionary role of aesthetic objects and events. Once it is clear what aesthetics is and what makes aesthetic objects and events different from other objects and events, I will explain some necessary restrictions and requirements of neuroaesthetic research.

AESTHETICS & ART

In order to address any topic in the philosophical field of aesthetics it is necessary to understand what exactly is meant by the term "aesthetics", and so it follows that defining aesthetics is also essential in the field of neuroaesthetics. When I use the term aesthetics I am referring to the acts of creating art, experiencing art and the artwork itself. By art I mean any work created by a human and expressed through some medium such as music, painting, dance, cinematography, literature, etc. Importantly, the definitions I am using for "aesthetics" and "art" do not exclude objects and events that are not created by humans; it is certainly possible for naturally occurring objects and events to be experienced as aesthetic objects and events. The viewpoint expressed in this paper both supports and allows objects and events not created by man or with a creative purpose to achieve the same cognitive effects as an aesthetic work intentionally created by man; the only requirement is that the object or event be thought of in an aesthetic context. The concept of the "aesthetic context" refers to a particular method of engaging with an object or event that is characterized by the use of a multitude of functionally different cognitive domains. The concept of the aesthetic context is crucial to any understanding of art, but before it can be addressed, the evolutionary foundations for such a cognitive process must be investigated.

If we are to appreciate the role aesthetics takes in the human species and also address the limitations inherent in the neuro-scientific study of such a role, we must first address the causal factors that brought about such a phenomenon. There are several key points regarding the de-

velopment of aesthetics in human cultural that need to be introduced. (1) Aesthetics is not a single cognitive event, but uses many different cognitive domains. (2) Aesthetics is not the result of one exclusive aesthetics adaption, music adaption, painting adaptation, etc. (3) Aesthetics does not need to have an adaptive purpose, or at least a simple and straightforward one. (4) Aesthetics is a product of the human brain.

Aesthetics utilizes the neurological networks already laid down by millions of years of evolution. For example, imaging studies have shown that simply listening to music activates:

> [A] widespread bilateral network of frontal, temporal, parietal and subcortical areas related to attention, semantic and music-syntactic processing, memory and motor functions, as well as limbic and paralimbic regions related to emotional processing. (Sarkamo et al., p. 867)

Natural selection never selected for specific aesthetic phenotypes and a random genetic mutation did not result in complex auditory processing capabilities, complex visual processing capabilities, complex motor control, memory capabilities, emotional capabilities, executive systems capable of planning series of events (such as playing a melody or rhythm), or systems which provide the basis for social interactions. The great variety and functional specificity of these different areas of the brain owe their own developmental trajectories to their existence and development as a part of the whole. The brain is vastly interconnected, and the development of one function influenced the development of other functions as well. The immense recruitment of a vast array of cognitive modalities utilized to perceive and create aesthetic stimuli may come across as a daunting challenge to fully understand. However, the cognitive processes previously mentioned are but a few of the building blocks necessary for aesthetic objects and events to be created, processed and interpreted the way they are today.

Non-biological factors also contributed to the development of aesthetics. Cultural aspects of language, social interaction, and tool use all served as non-biological catalysts for the development of aesthetic objects and events. Many researchers have attributed the origin of music to methods of building social trust (Roederer, 1984) or the verbal, music-like interactions between a mother and child (Dissanayake, 2009). Both of these possibilities and many more are likely to be true, but only in the sense that these non-biological factors played a part in the development of aesthetics. Aesthetic objects and events, such as music, arise from multiple contributors, both biological and non-biological. The recipe for aesthetics seems to consist of biological building blocks and a catalyst resulting from many non-biological contributions.

While the distinction between the biological and non-biological may seem clear, it is important to realize that in reality, the boundary is much fuzzier than I have made it out to be. Genetic and environmental factors in human development are immensely dependent upon each other. A clear example of the multiple factors involved in human development can be seen in the complex relationship between the genotype and phenotypes of any organism. My goal for this section was not to separate the biological factors from the non-biological factors, but to simply acknowledge that there is a difference, one that is crucial for the field of neuroaesthetics.

AESTHETIC CONTEXT

Art is a distinctive class of stimuli in a similar way that tools are a distinctive class of stimuli. What separates tools from other stimuli humans encounter is the way in which we understand and interact with them. Instead of understanding or thinking about tools strictly in terms of their sensory properties, humans seem to also code tools based upon the possible ways one can interact with them. This method of coding is referred to as an affordance. "Affordances reflect the structural properties of specific objects in that certain structural properties of objects imply certain usages" (Ward, p. 169). So the handle of a hammer implies its use as a hammer and is associated with the motion of hammering. As a result, this knowledge becomes an intimate part of the semantic category of hammers through what is termed an affordance. For example, studies of patients with lesion damage resulting in a certain form of semantic dementia have shown that these patients are capable of copying actions performed by others and using tools they had never used before. However, the same patients were unable to interact with tools they had used in the past, before the lesion occurred (Hodges et al., 2000). The semantic deficits of these patients imply that

affordances have unique neural correlates. In other words, tools are represented uniquely in the brain.

It is unlikely that tools were always represented as affordances since the very first instance of their use. The development of affordances for tools involved many biological and non-biological building blocks just like aesthetics, such as useful, naturally occurring objects and the cognitive ability to use one object to accomplish a task. With the increasing use of these naturally occurring objects to achieve a goal, humans began to distinguish these objects from others that served a distinguishable purpose. Eventually the use of objects to accomplish a task occurred often enough that the plasticity of the brain (not the evolution of the brain) resulted in affordances. The main point is that the building blocks of what we think of as tools existed in the human environment before tools existed and the way we think of tool stimuli changed.

In a similar manner, a wide range of pre-art objects and processes such as early language, tool use, and symbolic representation existed before aesthetics. These pre-art processes influenced the plastic development of human brains, which in turn led to what uniquely characterizes art. The plasticity resulted in increased interconnectivity between different brain regions and consequentially, the way the brain was capable of responding to certain stimuli changed. This change was the beginning of perceiving and interpreting certain objects and events in an aesthetic context. The change was not a gradual process, but eventually, the cultural products of these pre-art processes were perceived differently by the brain compared to other objects and events and this, in turn, affected the way these objects and events were performed and created. The plasticity of the human brain allowed for the functional connectivity between many distinct areas to be taken advantage of, allowing for emotions, ideas and pleasure to be brought forth by sensory stimuli. The way humans interpret, think about, and process aesthetic objects and events in modern cultures is the result of the plasticity of the brain, not strictly the evolution of the brain.

The example of affordances lends itself usefully to the development of aesthetic objects and events for two reasons. First, affordances developed as a result of the plasticity of the brain, and in the same way, the ability to perceive and interpret objects and events in an aesthetic context also developed as the result of the plasticity of the brain, not because of an evolutionary adaption specifically selecting for aesthetic capabilities. However, it should be noted that I am referring to different kinds of plasticity in each example. The plasticity required for the development of affordances as neural representations of objects involved a population of neurons developing a response to certain characteristics associated with certain objects. The plasticity which helped give rise to aesthetics involved increased dendritic branching and neurogenesis, resulting in structural plastic changes of the brain in the form of functional connectivity between different cognitive regions. These changes did not occur because of aesthetics, but were a result of the continuing development of the cognitive functions of the human brain in response to complex environments, and only after the functional connectivity existed in the brain, did aesthetics begin to resemble what is does today.

Second, both tools and aesthetic objects and events are interacted with differently compared to other objects as a result of the plasticity of the brain, not because of a pure semantic distinction based upon physical appearance. However, tools and aesthetic objects are different because tools are likely to always be perceived as tools in any context because what makes it a tool (affordances) is inherent in the structure of the object. Aesthetic objects are less likely to always be perceived as aesthetic objects because what characterizes them is not inherent in the object themselves, but the aesthetic context in which the viewer perceives it. Aesthetic objects are intentional in the sense that a viewer intentionally perceives an aesthetic object in the aesthetic context. Nothing in an aesthetic object requires that it be viewed in the aesthetic context.

It is not the structural properties of music, for example, that afford for a certain type of processing, but the cultural properties and assumptions of music along with the categorical similarity between encountered music and past exemplars. As a result, the recruitment of various cognitive domains in response to an aesthetic object is initiated by the viewer or creator of art both consciously and unconsciously. I mean "unconsciously" in the sense that because of cultural influences we automatically interpret some things as art and do not consciously think, "here is a piece of art, let me relate it to past experiences, emotions, etc.". The use of the aesthetic context by viewers and creators of art is inspired by both personal interest and the role of art in culture. For example, it is more likely that a

person will use complex networks of neurons to contemplate the meaning and associations inherent in a painting if it is encountered in a museum or gallery. Perhaps the viewer will be reminded of childhood trips to the beach with family when contemplating a painting of a sunset at an art exhibition, while the same painting hanging in the lobby of a dentist's office may simply provide an adequate distraction to the boring wallpaper. In the latter instance the painting may still be categorized as "art" (after all, someone probably intentionally created it for that purpose), but the engagement of many different cognitive domains that is unique to an aesthetic experience is absent. In the former instance the viewer engages with the painting by contemplating many different aspects and in the process, recruits many different cognitive domains. The first situation is an example of the application of the aesthetic context. Thus, when I say someone is "perceiving an object in an aesthetic context", aesthetic context is referring to a particular method of engaging with an object or event that is characterized by the use of many functionally different, but interconnected cognitive domains.

It is crucial to recognize that the aesthetic context is not an on-or-off state of mind, it is better thought of as a continuum. The interconnectivity of the brain is not only used for aesthetic experiences, but is also involved in every day life. We react emotionally to events every day. We can feel pain and are reminded of past failures if we fail a test, or feel joy and are reminded of childhood when we eat a favorite meal. However, if the failed test was worth 5% of a total grade compared to 50%, we may react a little less terrified and perhaps the incident will not conjure up gigantic failures of the past. Similarly, if the meal was a little blander we may experience satisfaction, or even happiness, but not joy. The same gradation in response can occur with aesthetic stimuli because the environment and countless other contingent factors can affect the extent to which a person engages with, for example, a movie or opera. The extent to which someone engages with an aesthetic stimulus is directly related to the use of the aesthetic context because nothing in the physical object demands the use of the aesthetic context to view it. The aesthetic context is a cognitive action taken toward the perception of the object or event, and there is no absolute degree to which a person must engage with an object for it to become aesthetic.

COGNITIVE FOUNDATIONS

Before the limitations inherent in a neuro-scientific study of aesthetics can be addressed an important and useful property of the human aesthetic experience must be considered. Individual neurological aspects of perceiving and creating aesthetic objects and events perform the same whether they are involved with aesthetic objects or non-aesthetic objects. The response of the neurons in the visual cortex will be the same regardless of whether the person is viewing a painting or navigating through a cafeteria at work. Similarly, the response of the neurons in the auditory processing areas will be the same regardless of whether the person is listening to their favorite music or listening to a professor's lecture. I do not mean to claim that the same response patterns will be produced by the two different situations, I simply mean that the basic sensory neurons will still be responding to the stimuli they have developed to respond to, regardless of the context. Remember that what does differentiate the neurological response to an aesthetic experience from that of any other cognitive act is the use of a multitude of functionally connected cognitive domains. The response characteristics of sensory and even the executive and memory systems will still serve the same function as they do for any other task outside of aesthetics.

Experiencing and creating aesthetic objects and events utilizes the same neurological systems that the brain uses to interact with all stimuli. As a result, research from other fields can all be considered in an aesthetic context and provide the same information that studying those processes in an aesthetic context in the first place would produce. For example, the knowledge gained by mapping the response characteristics of neurons in the visual cortex can be used to understand the visual processing of paintings, film and other art characterized by the visual properties of the stimuli. The auditory processes involved in perceiving music and theater can be understood from previous research on the response characteristics of neurons in the primary auditory cortex. Researchers have spent years studying the motor cortex and the executive functions of the frontal lobe and the knowledge gained can shed light on the motor processes and executive functions involved in creating a painting or musical piece.

Many researchers addressing aesthetic issues already use this approach. Ramachandran and Hirsteins' The Science of Art: A Neurological Theory of Aesthetic Experience (1999) attempts to use previously discovered response properties of the visual cortex such as the peak-shift effect, perceptual grouping/binding, and contrast extraction to explain certain characteristics of art. In her paper Neuroaesthetics: Neuroscientific Theory and Illustrations from the Arts (2008), Nalbantian discusses the role of neurons in the prefrontal cortex and hippocampus have in experiencing and creating art. In Zeki's paper entitled Neural Concept Formation in Art (2002), he attempts to explain works of Dante, Michelangelo and Wagner in neurological terms by tracing the origins of their works "to a fundamental characteristic of the brain, namely its capacity to form concepts" (p. 2). In summary, a great deal of information on the neural networks involved in experiencing and creating aesthetic objects and events can be obtained by research from other fields and does not require experimentation probing the same activations in the aesthetic context. With this foundation, we now move on to the limitations of neuroaesthetics.

LIMITATIONS: I

Now that we have established the nature of aesthetics, we can present some consequential limits to a neuro-scientific approach to the study of aesthetics. The first limitation to a neuroscience approach to the study of aesthetics is that, as stated earlier, part of what characterizes aesthetics is non-biological. This limitation exists to varying degrees because the effects of culture can manifest themselves on different levels. In their article The Science of Art: A Neurological Theory of Aesthetic Experience (1999) Ramachandran and Hirstein claim that a veiled nude is much more aesthetically pleasing than a completely nude figure because of the sharp contrast created by jewelry or cloth next to smooth skin. The authors then go on to suggest a neurological reason for the supposedly universal disposition to find veiled nudes more attractive than non-veiled nudes. The reason is that the attention grabbing effect of contrast plays an important role in nature, such as its use to camouflage predators and prey. Ramachandran and Hirstein's conclusion is that contrast can be rewarding and as a result, aesthetically pleasing.

The authors make two assumptions in their theory that do not seem to be true. First, they assume that finding a veiled nude more attractive than a non-veiled nude holds true across all cultures. This assumption seems to be entirely false. In his response to Ramachandran and Hirstein entitled What Information is Given by a Veil? (1999), Lanier provided evidence that veiled nudes are not always perceived as more attractive, stating:

> It is precisely in ancient India that a female nude figure without ornament was considered imperfect. The philosopher Carvaka compares an 'unornamented' literary sentence to the naked body without ornament, both being imperfect or 'unbeautiful'. On the other hand, in Classical West, only a completely nude figure could qualify for the highest ideal of purity; the image of a female nude wearing ornaments would at once make it obscene, as we know from early pornographic photographs (p. 65).

Second, they assume that a basic response characteristic of primary visual cortex neurons (increased response to contrast) is responsible for veiled-nudes being more attractive. But if veiled nudes are not more attractive in every culture, how can the neurological property that contrast is rewarding possibly explain the phenomena if everyone, regardless of culture, develops the same response characteristics in their neurons of the primary visual cortex? It is possible that the authors simply hypothesized the wrong neurological cause for the phenomenon, however, any proposed neurological cause will still suffer from the flaw that the veiled nude is simply not always experienced as more attractive than the non-veiled nude. The evidence suggests that while there may be some basic response properties of neurons in the visual cortex that influence what we derive pleasure from visually, top-down influences such as culture or personal taste also play a large role.

Ramachandran and Hirstein's article attempts to explain an aesthetic phenomenon in terms of neurological theory, but fails to acknowledge the limitations of such an approach to aesthetics. The authors failed to realize that non-biological influences can affect our response to art, not just response properties of neurons. However, these non-biological influences can successfully be investigated if the study approaches the hypothesis appropriately. For example, in language processing, it has been shown that

different languages still utilize the same basic underlying neurological structure for reading, although different aspects of these systems may be used differently based on the specific demands of the language (Ward, 2006). Chinese logographs are different from English words because logographic languages are based on a one-word-one-symbol principle where as the English language requires several different graphemes to make a word. Imaging research has shown that reading Chinese logographs may require increased demands on brain regions involved in semantics when compared to English (Chee et al., 2000). Despite a common agreement that language appeared too recently to be a direct evolutionary adaptation (Christiansen & Kirby, 2003), cultural differences between languages can still be seen in the way the neurological underpinnings interact, but the general processing mechanism seems to be universal. So it seems possible that certain cultural differences in aesthetics can be studied on a neurological level, however, these differences must be more deeply rooted than others.

The differences I am referring to are differences that can fundamentally change the way one responds or interprets different stimuli. For example, Western music is characterized by its emphasis on tonal harmony while Indian music is more characterized by its emphasis on melodies and rhythms (Schmidt-Jones, 2010). Furthermore, the basic building blocks of music are fundamentally different between the two cultures. In Western music, the major and minor keys consist of the same relationships between notes respectively. No such relationship is found in Indian music. The interval pattern varies from one scale to another, which means that the relationships between the notes are different. Indian music also differs from Western music in the method by which instruments are tuned. Western music revolves around equal temperament, in which only octaves are pure intervals (have simple frequency ratios) and Indian music incorporates just intonation, in which the fifth and third scale degrees are pure intervals. This difference can present itself as a problem for Western musicians attempting to understand some Indian musical pieces and I am suggesting that these differences are so fundamental to one's understanding of music that they may manifest themselves in a way similar to the differences in reading Chinese logographs and English letters.

In summary, I am claiming that the differences between Western music and Indian music are both phenomenologically different and neurologically different. A Western musician experiences Indian music differently than an Indian musician and an Indian musician experiences Western music differently than a Western musician because the development of their brain involved very different non-biological factors. If these differences are comparable to the differences between Chinese logographs and English, then not only will the experience be different, the neural correlates will differ too.

Non-biologically induced differences across cultures can be studied in the field of neuroaesthetics, but only when these differences fundamentally change the way one experiences and interacts with a stimulus. Most differences in aesthetic objects and events between various cultures will not reflect fundamental changes in the neurological processing, but simply fads tied to a culture's social/economic trends and philosophical/moral beliefs. These fads are analogous to differences in interests seen in most individuals throughout a lifetime. A young boy may be interested in becoming a professional baseball player some day and derive much satisfaction from playing the sport. But later in life, the same boy may decide he enjoys cooking and derive much satisfaction from preparing and eating food. It is possible that the differences may present themselves on a neurological level because of the demands on different sensory modalities (such as smell) or motor capabilities (throwing a pitch), if one's interest is invested in for a long enough time, but the reason for the change in interest is not likely to be found on a neurological level.

When attempting to explain an aesthetic phenomenon with neurological evidence one must first consider if the phenomenon is seen universally, in all cultures, and secondly, if the phenomenon is so deeply rooted in the particular culture that it would change the way one processes and thinks about certain stimuli. Therefore, the first limit to neuroaesthetics is that not everything that constitutes an aesthetic work can be explained or investigated on a neurological level.

LIMITATIONS: II

While some aspects of perceiving and creating art do not

have precise neurological explanations, some aspects can be usefully researched on a neurological level. However, experiments that attempt to uncover information unique to aesthetic experiences by using aesthetic stimuli are likely to encounter many confounds. For example, in the paper the Neural Correlates of Beauty (2004), Zeki and Kawabata attempt to examine the neurological underpinnings of a painting being perceived as beautiful. The results show an increase in activation in the orbito-frontal cortex for beautiful paintings and an increase in activation in the motor cortex for non-beautiful or ugly paintings. Unfortunately, their results suffer form a two-part problem. First, what has made beauty a center of philosophical debate for centuries is the seemingly subjective nature of perceiving beauty in an object or event. While it may be argued that true beauty is not subjective (Kant, 1995), the problem is that Zeki and Kawabata certainly did not attempt to verify the nature of the supposed "beautifulness" perceived by the subjects in the study. Instead, the subjects were presented with 300 different paintings on a computer monitor and asked to categorize them as ugly, neutral and beautiful. The "beautiful" paintings presented to the subjects while in the MRI scanner were then chosen according to what the individual participants identified as beautiful. As a result, it is not known whether or not the subjects were simply rating the more attractive stimuli as "beautiful" because the only other options were neutral and ugly. Second, even if beauty was perceived as a result of a painting presented to a subject, the beautifulness experienced cannot be remotely close to the response evoked when the original painting is viewed. In her response to Ramachandran and Hirsteins' article (1999), Kindy states that:

> One can never understand the all-encompassing, radiant atmosphere of a Mark Rothko painting, for example, unless standing in front of it. The scale alone of a Rothko canvas is meant to relate directly to the body, so that the painting can be 'absorbed' by more than the eyes. It is a directly physical experience. Looking at a reproduction is meaningless (p. 61).

While it may not be completely meaningless, looking at a reproduction is certainly not the same as looking at the original, or even a reproduction similar in size, etc. So, it is highly likely that the beauty perceived in response to the paintings shown on a computer monitor Zeki and Kawabata observed are not at all similar to the beautifulness perceived when experiencing the original paintings.

The situation in this experiment is similar to the example previously presented about the different ways a painting in an art exhibition and the same painting in a dentist's office are likely to be engaged by a person. While visiting an art exhibition, a person is more likely to fully engage with the painting and allow it to have cognitive effects far from what a normal visual stimulus in the environment typically encourages. However, a person in a loud and cramped MRI scanner, or even a psychology building on a college campus, is likely to engage with the aesthetic stimulus differently. This brings us back to another important factor regarding the aesthetic context; it is best thought of as a continuum, not an on-or-off state of mind. Zeki and Kawabata may have been actually recording activity during an aesthetic response and the data and conclusions may still applicable, but they do not explain the full, in depth picture; they are not studying a true sampling of this unique cognitive ability.

Studying aesthetic experiences in a lab introduces confounds into the results. What makes aesthetics unique is the aesthetic context used to perceive and interpret the object, and different environments put constraints on the aesthetic context. Participants' reasons for viewing the art will differ and environmental factors like noise and the space constraints of a scanner can prove to be quite distracting. Even if the aesthetic context is adequately controlled for in an experiment, the physical differences between lab stimuli and the actual objects the stimuli represent will prevent researchers from truly sampling an aesthetic experience. For example, consider the difference between listening to a song on an expensive, surround sound speaker system and a pair of headphones. These differences are especially influential for the visual arts because of confounding variables such as size and the presentation of the stimulus on a monitor. As a result, the second limitation to a neuro-scientific approach to aesthetics is that, unless the actual stimulus can be used and freely experienced, the differences between the actual objects and stimuli will result in confounding variables.

AN EXPERIMENTAL PROPOSAL

While a great deal of useful information about the neural

networks involved in aesthetics can be obtained from other areas of research, aesthetic objects and events do ultimately distinguish themselves as a unique category when perceived in the aesthetic context. This means that neuroaesthetics does have the capability of discovering neural mechanisms involved in art perception and creation that are not activated by non-aesthetic objects and events. But because these unique neural mechanisms are distinguished by the increase in functional connectivity between different regions of the brain, neuroscience techniques such as functional magnetic resonance imaging must focus on the relationships between widespread areas of activation to truly explore what is unique to aesthetics. The following is an example of a well-designed neuroaesthetic experiment based upon previous research that is not directly linked to the study of aesthetics.

The paper When the Brian Loses Its Self: Prefrontal inactivation during sensorimotor processing (2006) by Goldberg, Harel and Malach provides convincing evidence that increases in sensory processing inhibits self-related cortical areas. Participants in the experiment underwent both a slow and rapid categorization task along with an introspection task. During the slow categorization task the participants were asked to categorize visual and auditory stimuli at a rate of one stimulus every three seconds. The rapid categorization task was the same as the slow except that stimuli were presented at triple the rate. During the introspection task the participants no longer categorized the stimuli, but instead were asked to self-introspect about their own emotional responses to each image or sound. The researchers found that the primary motor cortex and non-retinotopic object areas including the lateral occipital complex extending into the posterior fusiform and collateral regions increased in activity during all conditions. However, the rapid condition resulted in a significantly higher activation compared to both the slow and introspection conditions. The researchers also found that both the slow and rapid conditions resulted in the inhibition of self-related cortical areas such as the left superior frontal gyrus, inferior frontal cortex, and parts of the inferior parietal cortex. The conclusion was that "self-related processes are not necessarily engaged during sensory perception and can be actually suppressed" (p. 329). I suggest that this conclusion may not apply to the processing of objects and events in the aesthetic context and would make for an interesting neuroaesthetic experiment.

The self-related processing inhibited in the experiment by Goldberg, Harel and Malach have been linked to a range of functions such as attributing mental states to others (Schmitz et al., 2004) and internally cued responses (Gusnard et al., 2001). Since the aesthetic context recruits a widespread activation of various neural networks, it seems entirely possible that if the subjects had viewed paintings and heard excerpts of songs instead of the stimuli used, the inhibition that accompanied increased sensory processing may not have taken place. While viewing the paintings in the bore of a scanner will most likely make it difficult for the participants to truly perceive the painting in the aesthetic context and as a result, prevent data collected from sampling a true aesthetic experience, a well designed task may still allow the collection of data on the relationship between the sensory and self-processing areas in a context unique to aesthetics. Participants could be asked to categorize the paintings according to whether the artist is trying to express a sad, happy, angry, or confused mood based upon the individual style of each painting. Perhaps participants would contemplate how an artist could produce such lines and colors and what the artist meant by them, and as a result, recruit areas involved in self-related processing that are typically inhibited during sensory processing. The results could differ because introspection based on a simple object will not require the attentional demands of introspection based on a complex painting. The simple images of objects used in the original experiment may simply have been held in some kind of working memory while the introspection task occurred. The subjects were not actually reflecting on the visual properties, but instead on the semantic memory of the object. However, introspecting on a complex painting may require the constant attention and focus of the perceiver because the information in the painting is too complex to be held in working memory or too unique to have been previously encountered and categorized. This is just one possible reason the results could differ using aesthetic stimuli. It seems possible that even if the results don't show simultaneous activation in the sensory and self-related regions, a relationship between the two areas may be discovered that would not be seen in a simple categorization task. The relationship could be a back and forth style of activation as participants focus on the visual stimulus and then contemplate the relationship between what they are seeing and how another human

would produce the lines and colors to express a certain emotion. I suggest that a painting viewed in the aesthetic context could result in a pattern of activation not seen in the Goldberg, Harel and Malach experiment because of the recruitment of many functionally different neural networks that characterizes the processing of aesthetic objects and events.

I am presenting this hypothesis as an example of a neuroaesthetic experiment that successfully recognizes the limits and cautions of neuroaesthetic research. The hypothesis makes a prediction on the way functionally different areas of the human brain respond to aesthetic stimuli. The experiment avoids investigating a specific phenomenon of an aesthetic experience uniquely tied to cultural interest or any other non-biological factors that would not manifest themselves on a neurological level. Furthermore, the suggested experiment avoids the confounds inherent in presenting reproductions of aesthetic stimuli differing in size and context from the original because the suggested experiment does not make demands on the participants that are likely to function differently when a reproduction of the stimuli is viewed. There is a large difference between asking participants in an experiment to emotionally respond to a painting (like in the Zeki and Kawabata paper) and asking them to contemplate how such a painting was created. I am not suggesting that contemplating how an artist created the painting is always inherent in the aesthetic context, but I am suggesting the process of deriving meaning and feeling from a visual or auditory configuration is key to the aesthetic experience. Lastly, the hypothesis is based on research from other fields, but proposes the possibility of a unique interaction between different areas of the brain that may not occur outside of aesthetics. Consequentially, neuroaesthetics may actually be the best field of research such a hypothesis can be tested in.

CONCLUSION

A neuroscientific approach to aesthetics should investigate not only where in the human brain such interconnectivity takes place, but also how and why. By asking "how" I am proposing questions regarding the neural mechanisms responsible for the interconnectivity. How does hearing a certain melody or viewing a certain shade of color remind one of past experiences, which in turn results in an emotional response? How can we perceive patterns in a painting or the curves of a sculpture and form ideas regarding how the object was created? How can we appreciate the choreography of a ballet and infer the emotions and ideas implied by the movements? By asking "why" I am proposing questions regarding the reasons why the recruitment of various neurological networks is unique to aesthetic objects and events. Why are aesthetic experiences sought after and what makes them pleasurable? These are just a few of the questions a well-designed neuroaesthetic experiment may eventually be able to answer.

Ultimately, aesthetics may provide the best method of studying the interconnectivity of the brain. It is crucial that we understand individual functions of the brain before we can understand complex functions such as processing or creating aesthetic objects and events. However, it is also crucial to recognize that we cannot completely understand the function of an area of the brain until we also understand how it interacts with other areas. Aesthetics can provide unique situations through which researchers can understand the interconnectivity between different regions of the brain that characterizes the way we think as human beings. But in order to properly do so, it is important that the limitations of a neuro-scientific approach to aesthetics, and the caution such an approach requires, be understood. These limitations and cautions to neuroaesthetic research are the result of many biological and non-biological contributions to the emergence of aesthetics in the human species. As a result, any research in the field of neuroaesthetics should consider the following ideas:

(1) A great deal of information on the neural networks involved in experiencing and creating aesthetic objects and events can be obtained by research from other fields.
(2) Not everything that constitutes an aesthetic work can be explained or investigated on a neurological level.
(3) Unless the actual stimulus can be used, the differences between the actual objects and experimental stimuli will result in confounding variables.

Aesthetics is a truly unique human activity. The ability to perceive an object in the aesthetic context requires the engagement of the brain to a far greater extent than most

other human activities necessitate. Technological advances in brain imaging techniques have allowed researchers unprecedented access to the human brain and future technological breakthroughs will allow researchers interested in neuroaesthetics and other research fields to greatly improve the reliability and accuracy of data. The ultimate goal of any neurological study or philosophical inquiry is to better understand how the human mind works. Many of the philosophical debates of the past have now been addressed by modern science, but aesthetics still remains an elusive and daunting topic for many neuroscientists. However, by knowing the limitations inherent in addressing aesthetic phenomenon with neuroscience, I believe that successful and useful experiments can be carried out that will not only inform us on the nature of art and aesthetics, but also the human mind in general.

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