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The effect of nicotine on anti-VEGF therapy in a mouse model of neovascular age-related macular degeneration

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Abstract

Purpose—Evaluate the effect of nicotine on anti-VEGF therapy in the treatment of neovascular age-related macular degeneration (AMD).

Methods—One group of mice received nicotine in drinking water and the other group received water only. Choroidal neovascularization (CNV) was induced with a laser. Nicotinic acetylcholine receptor (nAChR) $\alpha 7$ expression was evaluated by immunohistochemistry (IHC). Bevacizumab or adiponectin peptide II (APNpII) was injected intravitreally on day 7 post-laser and the effects were evaluated on days 14 and 21. α -bungarotoxin was injected intraperitoneally on days 2–5 and its effect evaluated on day 14.

Results—Expression of nAChR $\alpha 7$ was 2–7 times higher between days 3 and 7 post-laser compared to naïve mice. In water fed mice, APNpII, bevacizumab, and α -bungarotoxin significantly reduced CNV size. In nicotine fed mice, treatment with APNpII or bevacizumab did not significantly reduce CNV size, whereas α -bungarotoxin did have an effect. Comparing water and nicotine mice, CNV size was 61–86% smaller in water mice except for the α -bungarotoxin group where there was no difference. PDGF and VEGF expression was 1.5–2.5 fold higher at day 14 in nicotine treated mice.

Conclusions—Nicotine significantly blocks the effect of anti-VEGF therapy in the treatment of laser induced neovascular AMD. nAChR $\alpha 7$ is significantly up-regulated during the formation of CNV and treatment with a nAChR $\alpha 7$ antagonist decreases CNV size irrespective of nicotine administration-

Keywords

Adiponectin; age-related macular degeneration; Bevacizumab; choroidal neovascularization; mouse model; nicotine acetylcholine receptor; platelet derived growth factor; smoking; vascular endothelial growth factor; α -bungarotoxin

Introduction

Age-related macular degeneration (AMD) is the number one cause of legal blindness in those over 55 years old in the developed world and the number three cause overall.¹ Right now about 2 million in the U.S are affected and by 2020 it is estimated that about 3 million

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will be affected with this disease.² There are two clinical subtypes of AMD, the non-exudative, or dry and the neovascular, or wet form. Neovascular AMD is due to the growth of abnormal new vessels under the retinal pigment epithelium (RPE) or subretinal space from the subjacent choroid, termed choroidal neovascularization (CNV). This form is less common but accounts for about 90% of severe vision loss from AMD.³ Many therapies have been developed over the years to treat neovascular AMD although there is no cure. The most promising of date are the vascular endothelial growth factor (VEGF) inhibitors. Pegaptanib (Macugen) and Ranibizumab (Lucentis) are FDA approved and Bevacizumab (Avastin) is being used off-label for the treatment of neovascular AMD.⁴⁻⁷ Currently both Bevacizumab and Ranibizumab are mainly being used in the U.S. Current trials are comparing the two but the available evidence suggests Bevacizumab is similar in efficacy to Ranibizumab in treating neovascular AMD.⁷

Many environmental and genetic factors have been extensively studied to find risk factors for AMD. The most important environmental positive association has been with cigarette smoking.² Three population based studies have strongly confirmed smoking as a risk factor for either development or progression of neovascular AMD.⁸⁻¹⁰ Former smokers even retain some of the risk as compared current smokers, but it is decreased about 50%.¹⁰ We found no specific studies that compared nicotine exposure to actual smoking, but two studies show nicotine exposure alone increases the size and severity of neovascular AMD in mice.^{11,12} Nicotine is responsible for activation of the nicotinic acetylcholine receptors (nAChR). Recently it has been shown that nAChR are expressed by vascular endothelial cells and that activation by nicotine directly stimulates neovascularization in tumors and atherosclerotic plaques.¹³ Inhibition of laser induced CNV in a mouse model with the non-specific nicotine receptor antagonists, hexamethonium and mecamylamine, has been evaluated and suggests stimulation of CNV size occurs through the nAChR and not just by other mechanisms such as oxidative stress.^{11,12} It has also been shown that nicotine causes an increase in VEGF expression in CNV and we know that intraocular levels are decreased after anti-VEGF treatment.^{14,15} Platelet derived growth factor (PDGF) has also been suggested to be affected by nicotine and may play a role in the pathogenesis of CNV as well.¹⁶ What has not been shown though is the effect of nicotine and anti-VEGF treatment on both VEGF and PDGF levels in the CNV.

We know nicotine causes an increased risk of CNV in humans and in the mouse model increases the size and severity of CNV.¹¹ Nicotine appears to cause this by non-neuronal activation of the nAChR. In this study we aimed to evaluate the effect of nicotine on anti-VEGF therapy in the treatment of neovascular AMD.

Methods

Mice

Mice were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Male C57BL/6 mice – 6–7 weeks old were used (Jackson Laboratory, Bar Harbor, ME). This study was approved by the Institutional Animal Care and Use Committee (IACUC), University of Arkansas for Medical Sciences (UAMS), Little Rock, Arkansas. Sixty two (62) mice were used in this study.

Nicotine administration

One half of the mice received nicotine (Sigma-Aldrich, St. Louis, MO. Product number N1019) in their drinking water (100 µg/ml) for 30 days prior to laser induction of CNV and this was continued afterwards until their sacrifice. This level is known to produce serum nicotine levels similar to that of chronic moderate smokers after 30 days of treatment.¹¹⁻¹³

The other half drank unaltered drinking water. Water intake and weight was evaluated regularly to ensure similar conditions in all mice.

Laser treatment

Laser induced CNV was performed as previously described.^{11,17–19} Briefly the mice were fully anesthetized and their pupils were dilated. Then CNV was induced by argon laser photocoagulation with a slit lamp delivery system (Lumenis Inc., Santa Clara, CA; spot size –50 μ m; duration - 0.05 s; power - 260 mW). Three laser spots were placed in each eye around the optic nerve.

Histological investigation and immunohistochemistry

The eyes were fixed in a 10% formalin solution (Sigma, St. Louis, MO) and embedded in paraffin. 5 μ m sections were stained with hematoxylin and eosin and used for immunohistochemistry (IHC) as described before.²² Rabbit polyclonal to nicotinic acetylcholine receptor alpha 7 IgG (ab10096, Abcam, Cambridge, MA), goat polyclonal antibody against VEGF, (R & D Systems, Minneapolis, MN) and rabbit polyclonal to PDGF BB IgG (ab21234, Abcam, Cambridge, MA) were used as primary Abs. AF488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR) were used as secondary Abs for immunofluorescent study of nAChR α 7. Vectastain ABC Elite kit and Vector VIP substrate (both from Vector laboratories, Burlingame, CA) were used to investigate VEGF and PDGF. Nuclei were counterstained with Vector Methyl green (Vector laboratories, Burlingame, CA).

Localization and evaluation of nAChR α 7 staining

Water fed mice were divided in 5 groups (n=7 mice in each group). One group was not treated (control group) but other animals were treated with laser and sacrificed at day 1, 3, 5 and 7 post-laser. Paraffin sections of the eyes with laser spots were stained for nAChR α 7 as described above. Images of RPE and choroid including laser injured areas were captured using ZEISS LSM 510 laser confocal microscope. Differential interference contrast (DIC) was used to identify structures (RPE, choroid and subretinal tissue). Green nAChR α 7 positive fluorescence was scored as described before¹⁸: 0 – no staining; 1 – faint; 2 – moderate; 3 – intense. All sections stained for nAChR α 7 were masked. Scored mean value was calculated for each laser-injured area separately for RPE cells, choroid and subretinal tissue including CNV. Subretinal tissue was scored only at day 5 and 7 post-laser.

Evaluation of VEGF and PDGF staining

Mice were divided in 8 groups (n=3 mice in each group). One half of the mice were fed with water and the rest consumed nicotine solution as described above. All mice were treated with laser photocoagulation. Intravitreal injections of Bevacizumab (groups 1–4) or APNpII (groups 5–8) were done at day 7 post-laser as described below. Mice from groups 1, 2, 5 and 6 were sacrificed at day 14 post-laser and from groups 3, 4, 7 and 8 – at day 21 post-laser. Eyes were fixed in formalin and 5 μ m sections were stained for VEGF and PDGF as described above. Images of spot area were captured using Olympus light microscope. Purple VEGF and PDGF positive staining in laser spots was scored from 0 to 3: 0 – no staining; 1 – faint; 2 – moderate; 3 – intense. All sections studied for staining were masked.

Intravitreal injection

On day 7 after laser induction both eyes from the mice were injected intravitreally with either 50 μ g/2 μ l of Bevacizumab (Avastin) or adiponectin (APN) peptide II. In our preliminary experiments we used 20 μ g and 50 μ g doses of Avastin. We observed better inhibition of CNV by 50 μ g. Therefore, all experiments were done by using 50 μ g Avastin.

APNpII has been shown previously to be anti-angiogenic.^{18,20,21} It has also been shown in mice to reduce the size of laser induced CNV and inhibit VEGF expression in the mouse model by using different doses.^{14,18,22} The mouse eye was decompressed with 27G needle by inserting the needle through the conjunctiva and sclera 1 mm behind the limbus. Microinjector UMP3 equipped with Nanofil syringe 100 μ L and 33G blunt needle (World Precision Instruments, Sarasota, FL) was used for injections. An injection of 2 μ L of solutions was performed in 10 seconds.

Investigation of α -bungarotoxin effect on CNV size

Mice were divided in 6 groups (n=5 mice in each group). One half of mice (groups 1–3) were fed with water and the rest (groups 4–6) consumed nicotine solution for 30 days as described above. All animals were treated with laser photocoagulation. Group 1 and 4 received intraperitoneal injections of PBS (50 μ L), groups 2 and 5 were treated with a selective inhibitor of the nAChR α 7 α -bungarotoxin (Tocris bioscience, Ellisville, Missouri) 0.05 mg/kg in 50 μ L. Intraperitoneal injections were performed once daily from days 2 to 5 post-laser. At day 7 post-laser groups 1, 2, 4 and 5 were treated intravitreally with 2 μ L of PBS and groups 3 and 6 were injected with 50 μ g in 2 μ L of APNpII as described above. Mice from groups 1 and 4 were sacrificed at day 7 post-laser and groups 2, 3, 5 and 6 were investigated at day 14 post-laser. CNV size was evaluated as described below.

CNV size evaluation

Mice were sacrificed and then perfused on days 7, 14, and 21 after laser induction of CNV with a PBS solution containing 50 mg/ml fluorescein-labeled dextran (FITC-Dextran, 2 million average mw, Sigma, St. Louis, MO). The eyes were harvested, fixed in formalin, and RPE-choroid-scleral flat mounts were prepared as previously described.^{17–19} Z-stack images were then captured using confocal microscopy (Zeiss LSM510). All samples for CNV size evaluation were masked. The volume of the fluorescein isothiocyanate (FITC)-dextran perfused vessels in the laser injured zones was measured with ImageJ software as described before³¹.

Statistical Analysis

For all our experiments we used number of animals necessary to detect differences between groups with power more than 0.8. To estimate number of animals for experiments we did power analysis with Russ Lenth's Balanced ANOVA power and sample size applet Version 1.65 (Java Applets for Power and Sample Size). Statistical analyses were performed using Statistica Software (StatSoft Inc., Tulsa, OK). Averages for each time point were calculated and data is presented as mean $\pm$ SEM. Comparison between groups was performed using a two-tailed Mann-Whitney U Test or ANOVA and significance was defined as $p < 0.05$.

Results

Expression of nAChR α 7 in RPE and choroid

In this study we looked at one particular subtype of nAChR, the α 7 subtype, and evaluated if there was a difference in its expression after CNV induction. In naive eyes, those without CNV, nAChR α 7 is present in RPE and choroid at very low levels (Figure 1). After laser photocoagulation, nAChR α 7 expression was dramatically increased at day 3 post-laser in the RPE and choroid ($p < 0.05$) compared to naïve mice (Figure 1). Increased levels of nAChR α 7 were also detected in RPE and choroid at day 5 and 7 post-laser. In the sub-retinal tissue there was a 7 fold higher ($p < 0.05$) expression of nAChR α 7 compared to choroid of naive mice eyes at days 5 and 7 post laser (Figure 1).

Effect of nicotine on CNV size after anti-VEGF therapy

Nicotine consumption significantly ($p < 0.05$) increased CNV size at day 21 post-laser in mice without specific anti-VEGF therapy compared to water fed mice (Figure 2). Intravitreal anti-VEGF treatment with Bevacizumab or APNpII significantly reduced ($p < 0.05$) size of CNV in water fed mice compared to PBS injected mice (Figure 2). APNpII reduced the size of CNV at day 14 by 84% and on day 21 by 63%. Bevacizumab reduced the size of CNV at day 14 by 93% and on day 21 by 79%. Nicotine administration diminished effect of anti-VEGF therapy. CNV size in nicotine fed mice was significantly higher at day 14 and 21 compared to water drinking mice (Figure 2). In nicotine fed mice, compared to baseline on day 7, APNpII reduced the size of CNV at day 14 by only 35% and by day 21, the size had actually increased by 11% over baseline. Bevacizumab reduced the size of CNV at day 14 by 45% and by day 21 only 23% less. Histological investigation of formalin fixed paraffin sections of laser spots stained with hematoxylin and eosin confirmed negative effect of nicotine on anti-VEGF therapy (Figure 3). Spot area and number of new vessels was increased in mice fed with nicotine.

Effect of nicotine on expression of VEGF and PDGF after anti-VEGF therapy

We investigated expression of VEGF and PDGF in laser spots of water and nicotine fed mice after intravitreal treatment with Bevacizumab and APNpII by IHC. Semi-quantitative scoring of the positive staining in laser spots showed that on day 14, seven days after treatment with either Bevacizumab or APNpII, VEGF expression was 2.5 fold higher in the nicotine treated mice (Figure 4). PDGF expression was 1.5 to 2 fold higher at this same time after treatment with either agent (Figure 5).

Effect of nAChR $\alpha 7$ antagonist on CNV size after anti-VEGF therapy

In water fed mice, compared to baseline size at day 7: α -bungarotoxin reduced the size of CNV at day 14 by 85% ($p < 0.05$). This shows in water fed mice the size of the CNV in all groups after anti-VEGF treatment or a nAChR $\alpha 7$ antagonist were significantly reduced when compared to baseline (Figure 6).

Treatment with α -bungarotoxin on day 14 though reduced the size of CNV by 67% ($p < 0.05$) showing that a nAChR $\alpha 7$ antagonist had a better effect than anti-VEGF treatment in nicotine fed mice in reducing CNV size. In contrast there was no significant difference in effect of α -bungarotoxin comparing nicotine to water fed mice, it was effective in both groups in decreasing CNV size.

Discussion

In multiple studies cigarette smoking has been shown to be the most important environmental risk factor for AMD.^{2,8-10} Nicotine, one of more than 4000 compounds found in cigarettes, is one of the most important causes of vascular injury.²³ We also know that nicotine alone increases the size and severity of neovascular membranes.^{11,12,14} Lastly nicotine is suggested to be angiogenic as well and that its effect on CNV size is mediated through the nAChR on vascular endothelial cells.^{12,24,25} What was not known is whether this has any effect on anti-VEGF treatment for neovascular AMD. The effect of nicotine on anti-VEGF therapy in AMD patients has not been investigated before. Thus, to the best of our knowledge, there is no publication available in this area. Our study shows importance of nicotine mediated pathways in pathogenesis of CNV and regulation of VEGF stimulated growth of choroidal vessels. Thus, our studies clearly show that the smoking significantly reduces the effect of anti-VEGF therapy. Bevacizumab is a humanized monoclonal antibody and the literature is controversial on its effect in the murine model.²⁶ Some studies have suggested it has an effect whereas others haven't.²⁶ Given this we decided to also inject

APNpII. As discussed previously this peptide is anti-angiogenic, antagonizes VEGF, and has been shown to reduce the size of neovascular membranes in a murine model.^{18,20–22} For these reasons APNpII is a possible other treatment for neovascular AMD but has not been tested in humans yet.²²

In the water fed group we showed that Bevacizumab and APNpII both reduced the size of neovascular membranes at days 14 and 21 after laser induction as expected. In the nicotine fed group though we showed there was only a small decrease in the size of the CNV at day 14 and by day 21 there was essentially no effect of either Bevacizumab or APNpII in the reduction of the size of the neovascular membrane. We found no significant difference between Bevacizumab and APNpII in reducing the size of the neovascular membranes in this study at any time point. We also showed, as previously published¹¹, that nicotine increases the baseline size of CNV with a maximal difference being at day 21.

In previous studies the nAChR $\alpha 7$ subunit has been found to be the dominant receptor in nicotine induced cell signaling.^{24,25,27} Previous studies have also shown a inhibition of CNV formation with the non-specific nicotine receptor antagonists, hexamethonium or mecamylamine.^{11,12} What is not known though is whether nAChR is up-regulated any during CNV formation or if a specific inhibitor of the $\alpha 7$ subunit would have any effect on CNV size. Involvement of specific nicotinic receptors in regulation of VEGF expression by RPE cells and pro-angiogenic effect was discovered recently.^{28–30} What we found was that in naive mice eyes, with no CNV present, nAChR $\alpha 7$ is present throughout the retina at very low levels. After laser induction though, in all levels of the retina, it is highly up-regulated. This up-regulation is most substantial in the sub-retinal area where the bulk of the CNV is present. Overexpression of nAChR $\alpha 7$ may explain sensitivity of CNV growth to nicotine.

When comparing nicotine to water fed mice there was a significant difference at all time points for both Bevacizumab and APNpII in the size of the neovascular membrane. Since we found a significant increase in nicotine receptors (nAChR $\alpha 7$) after laser induction of CNV, it suggests this maybe one of the mechanisms by which nicotine increases the size and severity of CNV lesions. There are just more nicotinic receptors present to be activated when nicotine is present in higher levels such as the case with smokers. It also may explain why anti-VEGF therapy is not as effective in nicotine treated mice because another angiogenic receptor is up-regulated while VEGF is getting blocked. This is further suggested by the fact that inhibition of the nAChR $\alpha 7$ subunit causes a decrease in CNV size in not only water but nicotine fed mice as well. In fact treatment was significant with a nAChR $\alpha 7$ antagonist in nicotine fed mice whereas anti-VEGF treatment had little effect. We also did experiments looking at VEGF and PDGF levels with nicotine present and the effect of anti-VEGF treatment on those levels. It is known from a previous study that VEGF is up-regulated in CNV after nicotine exposure.¹⁴ In this study we looked the effect of anti-VEGF treatment on these levels. Our data seems to suggest that when nicotine is present in high levels, that VEGF and PDGF take longer to be decreased after anti-VEGF treatment. Whether this is simply because there is more VEGF or PDGF present with nicotine exposure or nicotine antagonizes the effect of anti-VEGF therapy is not known at this point. This though gives another reason for why anti-VEGF therapy has less of an effect on CNV when nicotine is present.

In conclusion, we know nicotine makes neovascular membranes worse in a mouse model and probably mediates this through the nicotinic acetylcholine receptor (nAChR $\alpha 7$). We showed in our study a potent effect of Bevacizumab on neovascular membrane size in water fed mice and again showed a potent effect of APNpII in the same. We also showed a significant up-regulation of nAChR $\alpha 7$ after laser induction of the neovascular membrane and a significant decrease in CNV size when treated with a nAChR $\alpha 7$ antagonist in both

nicotine and water fed mice. Lastly it also appears that VEGF and PDGF may remain present longer after anti-VEGF treatment when nicotine is present. This all gives reason why we found that when nicotine is present, anti-VEGF treatment (both Bevacizumab and APN peptide II) is not as efficacious in the treatment of neovascular AMD in the mouse model. This hopefully gives current smokers both with and without AMD another reason to quit, given that our only real treatment for neovascular AMD doesn't work as well or at all in current smokers. This also reaffirms that blockage of nicotine receptors (nAChR) and possibly selective blockage of the alpha 7 subtype (Figure 7) may be a new therapeutic option for treatment of this disease alone or in combination with anti-VEGF therapy.

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References

1. Cong R, Zhou B, Sun Q, Gu H, Tang N, Wang B. Smoking and the Risk of Age-related Macular Degeneration: A Meta-Analysis. *Annals of Epidemiology*. 2008; 18(8):647–656. [PubMed: 18652983]
2. Friedman DS, O'Colmain BJ, Muñoz B, et al. Eye Disease Prevalence Research Group. Prevalence of age-related macular degeneration in the United States. *Arch Ophthalmol*. 2004; 122(4):564–572. [PubMed: 15078675]
3. Ferris FL 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol*. 1984; 102:1640–1642. [PubMed: 6208888]
4. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2006; 355:1419–1431. [PubMed: 17021318]
5. Gragoudas ES, Adamis AP, Cunningham ET Jr, et al. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med*. 2004; 351:2805–2816. [PubMed: 15625332]
6. Weigert G, Michels S, Sacu S, et al. Intravitreal bevacizumab (Avastin) therapy versus photodynamic therapy plus intravitreal triamcinolone for neovascular age-related macular degeneration: 6-month results of a prospective, randomized, controlled clinical study. *Br J Ophthalmol*. 2008; 92:356–360. [PubMed: 18303156]
7. Ziemssen F, Grisanti S, Bartz-Schmidt KU, Spitzer MS. Off-Label Use of Bevacizumab for the Treatment of Age-Related Macular Degeneration: What is the Evidence? *Drugs Aging*. 2009; 26(4): 295–320. [PubMed: 19476398]
8. Klein R, Klein BEK, Linton KL, et al. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. *Am J Epidemiol*. 1993; 137:190–200. [PubMed: 8452123]
9. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. *Arch Ophthalmol*. 1996; 114:1518–1523. [PubMed: 8953988]
10. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. *Arch Ophthalmol*. 1996; 114:1193–1196. [PubMed: 8859077]
11. Suner IJ, Espinosa-Heidmann DG, Marin-Castano ME, et al. Nicotine Increases Size and Severity of Experimental Choroidal Neovascularization. *Invest Ophthalmol Vis Sci*. 2004; 45(1):311–317. [PubMed: 14691189]
12. Kiuchi K, Matsuoka M, Wu JC. Mecamylamine Suppresses Basal and Nicotine-Stimulated Choroidal Neovascularization. *Invest Ophthalmol Vis Sci*. 2008; 49(4):1705–1711. [PubMed: 18385094]
13. Heeschen C, Jang JJ, Weis M, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat Med*. 2001; 7:833–839. [PubMed: 11433349]
14. Kaliappan S, Jha P, Lyzogubov VV, et al. Alcohol and nicotine consumption exacerbates choroidal neovascularization by modulating the regulation of complement system. *FEBS Letters*. 2008; 582:3451–3458. [PubMed: 18789935]

15. Funk M, Karl D, Georgopoulos M, et al. Neovascular age-related macular degeneration: intraocular cytokines and growth factors and the influence of therapy with ranibizumab. *Ophthalmology*. 2009; 116(12):2393–2399. [PubMed: 19815292]
16. Grozio A, Catassi A, Cavalieri Z, Paleari L, Cesario A, Russo P. Nicotine, lung and cancer. *Anticancer Agents Med Chem*. 2007; 7(4):461–466. [PubMed: 17630920]
17. Espinosa-Heidmann DG, Suner I, Hernandez EP, Frazier WD, Csaky KG, Cousins SW. Age as an independent risk factor for severity of experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2002; 43:1567–1573. [PubMed: 11980875]
18. Bora PS, Kaliappana S, Lyzogubova VV, et al. Expression of adiponectin in choroidal tissue and inhibition of laser induced choroidal neovascularization by adiponectin. *FEBS Letters*. 2007; 581:1977–1982. [PubMed: 17466298]
19. Bora PS, Hu Z, Tezel TH, et al. Immunotherapy for choroidal neovascularization in a laser-induced mouse model simulating exudative (wet) macular degeneration. *Proc Natl Acad Sci*. 2003; 100:2679–2684. [PubMed: 12589025]
20. Brakenhielm E, Veitonmaki N, Kihara SCR, et al. Adiponectin-induced anti-angiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci*. 2004; 101:2476–2481. [PubMed: 14983034]
21. Fruebis J, Tsao TS, Avorschi S, et al. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci*. 2001; 98:2005–2010. [PubMed: 11172066]
22. Lyzogubov VV, Tytarenko RG, Thotakura S, et al. Inhibition of new vessel growth in mouse model of laser induced choroidal neovascularization by adiponectin peptide II. *Cell Biology International*. 2009; 33:765–771. [PubMed: 19422927]
23. Smith CJ, Hansch C. The relative toxicity of compounds in mainstream cigarette smoke condensate. *Food Chem Toxicology*. 2000; 38:637–646.
24. Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest*. 2002; 110(4):527–536. [PubMed: 12189247]
25. Cooke JP, Ghebremariam YT. Endothelial nicotinic acetylcholine receptors and angiogenesis. *Trends Cardiovasc Med*. 2008; 18(7):247–253. [PubMed: 19232953]
26. Yu L, Wu X, Cheng Z, et al. Interaction between bevacizumab and murine VEGF-A: a reassessment. *Invest Ophthalmol Vis Sci*. 2008; 49(2):522–527. [PubMed: 18234994]
27. Wu JC, Chruscinski A, De Jesus Perez VA, et al. Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. *J Cell Biochem*. 2009; 108(2):433–446. [PubMed: 19623583]
28. Pons M, Marin-Castaño ME. Nicotine increases VEGF/PEDF ratio in retinal pigment epithelium: a possible mechanism for CNV in passive smokers with AMD. *Invest Ophthalmol Vis Sci*. 2011 Feb 17. [Epub ahead of print].
29. Patil AJ, Gramajo AL, Sharma A, Seigel GM, Kuppermann BD, Kenney MC. Differential effects of nicotine on retinal and vascular cells in vitro. *Toxicology*. 2009 May 2; 259(1–2):69–76. [PubMed: 19428945]
30. Dom AM, Buckley AW, Brown KC, Egleton RD, Marcelo AJ, Proper NA, Weller DE, Shah YH, Lau JK, Dasgupta P. The $\alpha 7$ -nicotinic acetylcholine receptor and MMP-2/9 pathway mediate the pro-angiogenic effect of nicotine in human retinal endothelial cells. *Invest Ophthalmol Vis Sci*. 2011 Jun 22; 52(7):4428–4438. [PubMed: 20554619]
31. Lyzogubov VV, Tytarenko RG, Liu J, Bora NS, Bora PS. Polyethylene glycol (PEG) induced mouse model of choroidal neovascularization. *J Biol Chem*. 2011 May 6; 286(18):16229–16237. [PubMed: 21454496]

Figure 1.

IHC photographs showing relative staining for nAChR $\alpha 7$, which appears green. **A.** Expression of nAChR $\alpha 7$ in naïve mouse RPE-choroid. **B.** Expression of nAChR $\alpha 7$ in RPE-choroid and laser injured area at day 1 post-laser. **C.** Expression of nAChR $\alpha 7$ in RPE-choroid and laser injured area at day 3 post-laser. **D.** Expression of nAChR $\alpha 7$ in RPE-choroid and laser injured area at day 5 post-laser. **E.** Expression of nAChR $\alpha 7$ in RPE-choroid and laser injured area at day 7 post-laser. **F.** Merged image of DIC and IHC staining for nAChR $\alpha 7$ in naïve mouse RPE-choroid. **G.** Merged image of DIC and IHC staining for nAChR $\alpha 7$ in RPE-choroid at day 1 post-laser. **H.** Merged image of DIC and IHC staining for nAChR $\alpha 7$ in RPE-choroid at day 3 post-laser. **I.** Merged image of DIC and IHC staining for nAChR $\alpha 7$ in RPE-choroid at day 5 post-laser. **J.** Merged image of DIC and IHC staining for nAChR $\alpha 7$ in RPE-choroid at day 7 post-laser. **K.** A bar graph breaking down the intensity of nAChR $\alpha 7$ found in the RPE, choroid, and sub-retinal tissue. In the RPE and choroid on days 3, 5, and 7 and in the sub-retinal tissue on days 5 and 7, there is significantly more nAChR $\alpha 7$ present compared to naive eyes. Star labels laser spot or subretinal tissue including CNV. Bar = 40 μm .

Figure 2.

Confocal microscopy photographs of the FITC-dextran perfused CNV (green color). **A.** CNV of water fed and PBS injected mouse at day 7 post-laser. **B.** CNV of water fed and PBS injected mouse at day 14 post-laser. **C.** CNV of water fed and PBS injected mouse at day 21 post-laser. **D.** CNV of water fed and Bevacizumab injected mouse at day 14 post-laser. **E.** CNV of water fed and Bevacizumab injected mouse at day 21 post-laser. **F.** CNV of water fed and APNpII injected mouse at day 14 post-laser. **G.** CNV of water fed and APNpII injected mouse at day 21 post-laser. **H.** CNV of nicotine fed and PBS injected mouse at day 7 post-laser. **I.** CNV of nicotine fed and PBS injected mouse at day 14 post-laser. **J.** CNV of nicotine fed and PBS injected mouse at day 21 post-laser. **K.** CNV of nicotine fed and Bevacizumab injected mouse at day 14 post-laser. **L.** CNV of nicotine fed and Bevacizumab injected mouse at day 21 post-laser. **M.** CNV of nicotine fed and APNpII injected mouse at day 14 post-laser. **N.** CNV of nicotine fed and APNpII injected mouse at day 21 post-laser. **O.** Bar graph shows quantification of size of the CNV. In the control groups, at day 21, nicotine fed mice had a much larger area of CNV compared to water fed mice (* $p < 0.05$). In the Bevacizumab and APNpII treated groups, water fed mice had a significant reduction in the size of CNV after treatment at both days 14 and 21 (* $p < 0.05$). Comparing the nicotine to water fed mice, nicotine significantly reduced the effect of Bevacizumab and APNpII (# $p < 0.05$). Bar = 100 μm .

Figure 3.

Histological changes of CNV after Bevacizumab and APNpII treatment. Representative microphotographs show CNV complex with new choroidal vessels (star) at day 7, 14 and 21 after laser photocoagulation in water and nicotine fed mice. **A.** CNV of water fed and PBS injected mouse at day 7 post-laser. **B.** CNV of nicotine fed and PBS injected mouse at day 7 post-laser. **C.** CNV of water fed and APNpII injected mouse at day 14 post-laser. **D.** CNV of nicotine fed and APNpII injected mouse at day 14 post-laser. **E.** CNV of water fed and Bevacizumab injected mouse at day 14 post-laser. **F.** CNV of nicotine fed and Bevacizumab injected mouse at day 14 post-laser. **G.** CNV of water fed and APNpII injected mouse at day 21 post-laser. **H.** CNV of nicotine fed and APNpII injected mouse at day 21 post-laser. **I.** CNV of water fed and Bevacizumab injected mouse at day 21 post-laser. **J.** CNV of nicotine fed and Bevacizumab injected mouse at day 21 post-laser. Treatment with Bevacizumab and APNpII reduced the size of the CNV complex and number of vessels in the CNV complex by day 14 post-laser in water fed animals. Nicotine administration resulted in less reduction of CNV compared to water fed mice at day 14 and 21 post-laser. Bar = 20 μ m.

Figure 4.

Representative IHC photos showing expression of VEGF. Nuclei were counterstained with vector methyl green and appear bluish-green. Purple color indicates VEGF staining. **A.** VEGF expression in CNV of water fed mouse at day 14 post-laser. **B.** VEGF expression in CNV of water fed mouse at day 21 post-laser. **C.** VEGF expression in CNV of nicotine fed mouse at day 14 post-laser. **D.** VEGF expression in CNV of nicotine fed mouse at day 21 post-laser. **E.** Bar graph shows semi-quantitative evaluation of VEGF-positive staining in laser spots. VEGF expression decreased in water fed mice but not in nicotine treated mice on day 14. On day 21 expression of VEGF was similar in all groups.

Figure 5.

Representative IHC photos showing expression of PDGF. Nuclei were counterstained with Vector methyl green and appear bluish-green. Purple color indicates PDGF staining. **A.** PDGF expression in CNV of water fed mouse at day 14 post-laser. **B.** PDGF expression in CNV of water fed mouse at day 21 post-laser. **C.** PDGF expression in CNV of nicotine fed mouse at day 14 post-laser. **D.** PDGF expression in CNV of nicotine fed mouse at day 21 post-laser. **E.** Bar graph shows semi-quantitative evaluation of PDGF -positive staining in laser spots. PDGF expression decreased in water fed mice but not in nicotine treated mice on day 14. On day 21 expression of PDGF was similar in all groups.

Figure 6.

Bar graph showing the effect of α -bungarotoxin on CNV size. In nicotine fed mice, treatment with APNpII at day 14 had minimal effect but treatment with α -bungarotoxin had a significant effect in decreasing the size of CNV at day 14 (# $p < 0.05$). In control mice, both α -bungarotoxin and APNpII decreased the size of CNV at day 14 (* $p < 0.05$). There was no difference in the effect of α -bungarotoxin compared to nicotine and control mice.

Figure 7. Schematic mechanism of CNV growth regulated by Nicotine. Treatment with nAChR $\alpha 7$ antagonist inhibits nAChR $\alpha 7$ and leads to the reduction of growth factors and then inhibition of CNV.