UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Kaja PLIBERŠEK

ŽIVČNE POVEZAVE MED REGENERIRANIM VOHALNIM EPITELOM IN VOHALNIM BULBUSOM PRI AMERIŠKEM SOMIČU (*Ameiurus melas*)

DOKTORSKA DISERTACIJA

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NERVE CONNECTIONS BETWEEN REGENERATED OLFACTORY EPITHELIUM AND OLFACTORY BULB IN BLACK BULLHEAD CATFISH (*Ameiurus melas*)

DOCTORAL DISSERTATION

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Spodaj podpisana Kaja Pliberšek se strinjam z objavo moje doktorske disertacije na spletni strani Digitalne knjižnjice Biotehniške fakultete. Izjavljam, da je doktorska disertacija, ki sem jo oddala v elektronski obliki, enaka tiskani različici.

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Kaja Pliberšek

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AI Vohalni epitel vretenčarjev sestavljajo vohalne celice, ki so ob okušalnih celicah edine čutilne celice neposredno izpostavljene okolju. Mlade vohalne celice mesečno nadomeščajo stare vohalne celice, do nadomeščanja prihaja tudi po okužbah in mehanskih poškodbah. Pri ameriškem somiču (*Ameiurus melas*) smo izrezali vohalne rozete; v nosni votlini smo pustili majhne osrednje dele vohalnih lamel z delom rafe. Odvisno od obsega izrezanja vohalnih rozet so vohalni organi regenerirali v 3-20 mesecih. Da bi sledili aksonom iz regeneriranega vohalnega epitela do vohalnega bulbusa smo retrogradno, z uporabo fluorescentnega označevalca membran, DiI, obarvali vohalne celice. V primeru, ko smo v nosni votlini pustili dele dvanajstih do osemnajstih vohalnih lamel in dela rafe, so se regenerirani aksoni vohalnih celic povezali z istimi področji vohalnega bulbusa kot pri intaktnih vohalnih organih; zrasle so majhne popolne vohalne rozete ali majhne pahljačaste vohalne rozete. Intaktne in regenerirane vohalne rozete so vsebovale dolge vohalne celice s cilijami in vohalne celice srednjih dolžin z mikrovili. Regenerirni vohalni organi so se elektrofiziološko odzivali na aminokisline in so somičem omogočali njihovo razlikovanje (Stenovec, 2001). Iz majhnih delov treh do sedmih vohalnih lamel in rafe so v 80% primerov zrasle prstaste lamele ali nepravilni izrastki epitela in veziva, ki niso delovali kot vohalni epitel. V nekaj primerih (<20%) so pri teh ribah iz zarodnega epitela zrasle majhne vohalne rozete z 2-12 lamelami, ti vohalni organi so začeli delovati 8-20 mesecev po operaciji. Tak vohalni epitel je vseboval dolge vohalne celice s cilijami in vohalne celice srednjih dolžin z mikrovili, njihovi aksoni so konvergirali v ista območja vohalnega bulbusa kot pri intaktnih vohalnih organih. Majhne vohalne rozete so se elektrofiziološko odzivale na širok spekter aminokislin, celo na vohalno šibek dražljaj L-prolin. Somiči z enostransko regeneracijo majhnega vohalnega organa so razlikovali aminokisline. Pri popolni odstranitvi vohalnega organa smo odstranili tudi zarodne celice; regeneracija vohalnega organa ni bila mogoča.

KEY WORDS DOCUMENTATION

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AB Olfactory epithelia of vertebrates are composed of olfactory receptor neurons (ORNs) that are, in addition to taste cells, the only receptor neurons directly exposed to the environment. Old ORNs are replaced continuously approximately once per month with young ORNs, their regeneration also takes place after infection and mechanical injury. In the black bullhead catfish (*Ameiurus melas*) we partially excised olfactory rosetae, small portions of few olfactory lamellae and rapha were left in the nasal cavity; depending on the magnitude of the excision olfactory organs regenerated during 3-20 months. To trace axons from the regenerated olfactory epithelium to the olfactory bulb (OB) we retrogradey labelled ORNs with fluorescent marker DiI. If small portions of twelve to eighteen lamellae and part of the rapha were left in the nasal cavity during excision, the regenerated tall ciliated and intermediate microvillar ORN conneted to the same OB areas as in the intact olfactory organs; either small complete or fan-like olfactory rosetae were observed. These olfactory organs responded to amino acids electrophysiologically and enabled amino acid discrimination (Stenovec, 2001). If small portions of three to seven lamellae with a short section of the rapha were left *in situ*, either non-functional finger-like lamellae or irregular epithelial and connective tissues formed in most of the cases (80%). In less than 20% of the cases a miniature deformed olfactory rosetae containing 2-12 lamellae emerged; they became functional after 8 to 20 months. The miniature olfactory rosetae contained tall ciliated and intermediate microvillar ORNs converging into the same OB areas as in the intact olfactory organ. These olfactory organs were physiologically functional and they responded to wide range of amino acids, including the poorly stimulatory L-proline. Catfish with unilateral regeneration of only one miniature olfactory organ discriminated between amino acids. After excision of the olfactory rosetae that removed the progenitor cells, regeneration of the olfactory organ did not occur.

KAZALO VSEBINE

KAZALO SLIK

1 UVOD

1.1 ZGRADBA VOHALNEGA ORGANA VRETENČARJEV

Vohalni organ vretenčarjev vsebuje vohalne celice, ki z aksoni segajo v sprednji del telencefalona, v vohalni bulbus (Shepherd, 1991; Laberge in Hara, 2001). V vohalnem bulbusu tvorijo aksoni vohalnih celic sinapse z nevroni drugega reda, mitralnimi celicami. Področje, kjer končiči vohalnih celic tvorijo sinapse se imenuje glomerul. Pri cebricah (*Danio rerio*) je večina glomerulov kroglastih oblik, nekaj glomerulov tvori glomerularne preplete in glomerularne grozde (Baier in Korsching; 1994).

Vohalne celice na apikalni površini nosijo cilije ali mikrovili (Yamamoto, 1982; Laberge in Hara, 2001). Pri podganah so vohalne celice s cilijami del glavnega vohalnega organa, medtem ko vohalne celice z mikrovili sestavljajo vomeronazalni organ. Ribe nimajo vomeronazalnega organa, njihov vohalni organ vsebuje vohalne celice s cilijami in mikrovili (Lowe in MacLeod, 1975; Caprio in Raderman-Little, 1978; Cancalon, 1978, 1983a; Ichikawa in Ueda; 1977; Yamamoto, 1982; Hansen in Zeiske, 1998). Vohalni epitel rib vsebuje tudi kriptne celice, ki so verjetno tudi kemosenzorične (Hansen in Zeiske, 1998; Hansen in Finger, 2000; Hansen in sod., 2003). Kriptne celice na apikalni površini nosijo cilije in mikrovile.

1.2 ZGRADBA VOHALNEGA ORGANA RIB

Vohalni organ rib je parna struktura, ki leži na sprednjem dorzalnem delu glave. Gradita ga leva in desna vohalna kamrica, ki se odpirata v okolje z dotekalko in odtekalko (Zeiske in sod., 1992; Caprio in Raderman-Little, 1978). Na dnu vohalne kamrice leži naguban vohalni epitel. Gube vohalnega epitela, vohalne lamele, so pritrjene na osrednjo rafo, in skupaj z njo tvorijo vohalno rozeto (Slika 1). Večji del površine vohalnih lamel pokriva nečutilni epitel s kinocilijami. Te poganjajo vodni tok med vohalnimi lamelami (Caprio, 1988). Pri somičih je v osrednjem delu vohalnih

lamel, ob rafi, zaplata čutilnega epitela (Caprio in Raderman-Little, 1978). Čutilni epitel sestavljajo vohalne, podporne in bazalne celice. Pri kanalskem somiču (*Ictalurus punctatus*) in zlati ribici (*Carassius auratus*) aksoni vohalnih celic s cilijami in mikrovili ter aksoni kriptnih celic konvergirajo v različna območja vohalnega bulbusa (Morita in sod., 1996; Morita in Finger, 1998; Hansen in sod., 2003, 2005). Nedavna raziskava na cebricah je pokazala, da aksoni vohalnih celic, ki izražajo enega ali nekaj tipov vohalnih receptorjev, konvergirajo v prostorsko ločen grozd glomerulov (Sato in sod., 2007). Pri podganah vohalne celice, ki izražajo enake vohalne receptorje, konvergirajo v enega ali nekaj prostorsko ločenih glomerulov (Vassar in sod., 1994; Ressler in sod., 1994; Mombaerts in sod., 1996). Molekularno biološke raziskave so pokazale, da se na en tip vohalnega receptorja lahko veže več kot ena vonjava in da se ena vonjava lahko veže na več kot en tip vohalnih receptorjev (Krautwurst in sod., 1998; Zhao in sod., 1998; Speca in sod., 1999; Malnic in sod., 1999; Touhara in sod., 1999; Wetzel in sod., 1999; Araneda in sod., 2000; Luu in sod., 2004). V vohalnem bulbusu različne vonjave sprožajo različne vzorce glomerularnih aktivnosti (Costanzo in Mozell, 1976; Kauer, 1988; Friedrich in Korsching, 1997, 1998; Fuss in Korsching, 2001; Rubin in Katz, 1999; Uchida in sod., 2000; Nikonov in Caprio, 2001; Wachowiak in Cohen; 2001; Wachowiak in sod., 2002). Vedenjske raziskave na cebricah in ameriških somičih (*Ameiurus melas*) so razodele, da igra različnost vzorcev glomerularnih aktivnosti ključno vlogo pri razlikovanju vonjav (Valentinčič in sod., 2005). Cebrice in somiči razlikujejo amino kisline, ki prožijo različne vzorce glomerularnih aktivnosti, medtem ko aminokisline, kot sta L-valin in L-izolevcin, ki prožijo zelo podobne vzorce glomerularne aktivnosti, cebrice in somiči ne razlikujejo (Valentinčič in sod., 2000, 2005).

1.3 REGENERACIJA VOHALNEGA EPITELA IN NJEGOVIH POVEZAV Z VOHALNIM BULBUSOM

Mesečno se stare vohalne celice nadomeščajo z mladimi vohalnimi celicami, ki nastajajo iz bazalnih celic na dnu vohalnega epitela (Graziadei in Monti Graziadei, 1979; Suzuki in Takeda 1991; Schwob in sod., 1994; Caggiano in sod., 1994; Huard in sod., 1998; Ohta in Ichimura, 2001; Carter in sod., 2004; Schwob, 2005). Neprestano nadomeščanje starih vohalnih celic z novimi ima za posledico nenehno vzpostavljanje novih živčnih povezav med vohalnim epitelom in mitralnimi celicami v vohalnem bulbusu (Graziadei in Monti Graziadei, 1978, 1979). Vohalne celice se nadomeščajo tudi po mehanskih, kemičnih ali parazitskih poškodbah vohalnega epitela (Cancalon, 1982, 1983b; Hansen in sod., 1994; Valentinčič in sod., 2000; Iwema in sod., 2004) in po prerezanju vohalnega živca (Nordlander in Singer, 1973; Cancalon in Elam, 1980; Cancalon, 1987; Morison in Costanzo, 1989; Zelinski in Hara, 1992; Hoyk in sod., 1993). Če so bazalne celice vohalnega epitela uničene, ta ne regenerira (Byrd, 2000; Valentinčič in sod., 1994, 2000; Vankirk in Byrd; 2003; Costanzo, 2005). Raztopine cinkovega sulfata ($ZnSO_4$), detergenta Triton X-100 in nekaterih drugih kemikalij povzročijo propad vohalnih celic. Kemično poškodovan vohalni epitel po mesecu dni regenerira do prvotnega stanja (Cancalon, 1982, 1983b; Burd, 1993; Hansen in sod., 1994; Ducray in sod., 2002; Iwema in sod., 2004). Elektrofiziološke študije so pokazale, da po regeneraciji vohalnega organa ta ponovno deluje (Oley in sod., 1975; Bedini in sod., 1976; Simmons in Getchell, 1981; Evans in Hara, 1985; Zippel in sod., 199 3; Troitskaia, 1987). Pri šarenki (*Oncorhynchus mykiss*) je po prekinitvi vohalnega živca prišlo do začasne izgube elektrofiziološkega odziva; po 84 dneh je bil vohalni organ ponovno elektrofiziološko aktiven (Evans in Hara, 1985). Pri hrčku (*Mesocricetus auratus*) so pokazali, da po prekinitvi vohalnega živca aksoni mladih vohalnih celic ponovno dosežejo vohalni bulbus in vzpostavijo sinapse z mitralnimi celicami (Costanzo, 1985; Koster in Costanzo, 1996). Pri miškah so po prekinitvi vohalnega živca, aksoni vohalnih celic, ki so izražale P2 vohalni receptor, konvergirali v več kot en glomerul (Costanzo, 2000, 2005). Po kemični poškodbi vohalnega epitela miši so aksoni mladih vohalnih celic vzpostavili povezave v enakovrednih področjih vohalnega bulbusa, kot aksoni vohalnih celic v intaktnem vohalnem organu (Cummings in sod., 2000; Sengoku in sod.; 2001). Novejše raziskave kažejo da imajo

ovojne celice, ki so glia celice vohalnega sistema, in fibroblasti pomembno vlogo pri regeneraciji vohalnega organa (Williams in sod., 2004; Li in sod., 2005). Te celice vzdržujejo odprte poti med vohalnim epitelom in vohalnim bulbusom. Ovojne celice in fibroblasti ne regenerirajo skupaj z vohalnimi celicami (Li in sod., 2005).

1.4 OVOJNE CELICE VOHALNEGA ORGANA IN REGENERACIJA PERIFERNEGA ŽIVČNEGA SISTEMA IN HRBTENJAČE

Hrbtenjača in periferni živčni sistem omogočata refleksne odzive skeletnega mišičja in ritmična gibanja, kot sta hoja in plavanje (Hall, 2004). Povezave hrbtenjače z višjimi možganskimi centri omogočajo zapletene, zavestne gibe. Poškodbe perifernega živčnega sistema in hrbtenjače povzročajo paralizo (Ramon-Cueto in sod., 2000; Hall, 2004).

Hrbtenjača odraslega sesalca vsebuje zarodne celice, ki se lahko razvijejo v živčne celice, astrocite in oligodendrocite (Weiss in sod., 1996; Pincus in sod., 1998; Johansson in sod., 1999). Kljub prisotnosti zarodnih celic pri odrastlih in starejših mladih živalih regeneracija hrbtenjače po poškodbi ne poteče (Ramon-Cueto in sod., 1998; Bartolomei in Greer, 2000; Franklin in Barnett, 2000). Zarodne celice hrbtenjače se pospešeno delijo, diferencirajo v astrocite in prispevajo k tvorbi brazgotine (Johansson in sod., 1999). Poškodovani živci perifernega živčnega sistema, ki so izgubili pot med periferijo in hrbtenjačo, regenerirajo le delno in ne omogočijo prvotne funkcije (Bartolomei in Greer, 2000; Franklin in Barnett, 2000). Regenerirani čutilni živci dosežejo dorzalni rog hrbtenjače, vendar se vanj ne vrastejo in ne tvorijo sinaps (Franklin in Barnett, 2000). Regenerirani motorični živci se pogostokrat cepijo; veje živca se namesto v ciljne mišice nekje na periferiji vrastejo v bližnja tkiva (Verdu in sod., 1999).

Odkrili so, da vsaditev ovojnih celic vohalnega organa na mesto poškodbe hrbtenjače omogoča omejeno regeneracijo živčnih poti znotraj poškodovanega dela hrbtenjače (Li in sod., 1998; Ramon-Cueto in sod., 1998; Imazumi in sod., 2000; Ramon-Cueto

in sod., 2000; Lu in sod. 2001; Lu in sod., 2002; Ramer in sod., 2004). Delu paraliziranh podgan so se 3-10 mesecev po prerezanju hrbtenjače povrnili senzo-motorični refleksi, te podgane so nespretno hodile (Ramon-Cueto in sod., 2000; Lu in sod. 2001). Po poškodbah perifernega živčnega sistema so ovojne celice vohalnega organa z mielinskimi ovojnicami ovile aksone poškodovanih živčnih celic (Bartolomei in Greer, 2000; Franklin in Barnett, 2000). Senzorični živci so se v prisotnosti ovojnih celic vohalnega organa vrasli v dorzalni rog hrbtenjače, kjer je vsaj nekaj živčnih celic tvorilo sinapse (Franklin in Barnett, 2000). Poškodovani motorični živci se v prisotnosti ovojnih celic običajno ne cepijo (Verdu in sod., 1999). Ovojne celice delujejo kot usmerjevalci aksonov; regenerirani motorični živci so v prisotnosti ovojnih celic razmeroma pogostokrat dosegli tkivo, ki so ga oživčevali pred poškodbo. Delna regeneracija motoričnih živcev je v prisotnosti ovojnih celic vohalnega organa potekla celo v primeru, ko je po poškodbi 12-15 mm živca manjkalo (Verdu in sod., 1999).

Regeneracija perifernega živčnega sistema in hrbtenjače tudi v prisotnosti ovojnih celic vohalnega organa ni popolna (Bartolomei in Greer, 2000; Franklin in Barnett, 2000). Podrobnejše razumevanje mehanizmov regeneracije vohalnega organa, bi pripomoglo k razumevanju pogojev, ki bi omogočili uspešno regeneracijo živčnih celic v perifernem in centralnem živčnem sistemu (Bartolomei in Greer, 2000).

2 NAMEN DELA

Intaktni ameriški somiči razlikujejo skoraj vse aminokisline, medtem ko anozmični somiči aminokislin ne razlikujejo (Valentinčič in sod., 1994, 2000). Delno kirurško izrezanje vohalnih rozet ameriškega somiča povzroči začasno izgubo sposobnosti vohalnega razlikovanja. Te sposobnosti se povrnejo po regeneraciji vohalnih rozet (Valentinčič in sod., 2000). Celo zelo majhne regenerirane vohalne rozete pahljačastih oblik so se elektrofiziološko odzvale na aminokisline in so somičem omogočale popolno razlikovanje aminokislin (Valentinčič in sod., 2000; Stenovec, 2001). Med dosedanjimi raziskavami regeneracije vohalnega organa ameriškega somiča niso definirali obsega izrezanja vohalnega organa. Preverili smo, ali je velikost regeneranega vohalnega organa odvisna od površine vohalnega epitela, ki po operaciji ostane v nosni votlini. Predvidevali smo, da s popolno odstranitvijo vohalnega organa odstranimo zarodne celice in vohalni organ ne regenerira. Poleg tega smo iskali najmanjše regenerirane vohalne organe, ki somičem omogočajo razlikovanje aminokislin. Vohalne organe smo si ogledali pod svetlobnim in vrstičnim elektronskim mikroskopom. Z maščobnim označevalcem membran DiI smo preverili, ali se po regeneraciji obnovijo živčne povezave med regeneriranim vohalnim epitelom in vohalnim bulbusom. Uspešnost regeneracije vohalnih organov smo ugotavljali z elektrofiziološkimi meritvami (elektroolfaktogram, EOG) in vedenjskimi testi, pri katerih smo somiče naučili razlikovanja aminokislin.

3 MATERIALI IN METODE

3.1 POSKUSNE ŽIVALI

Ameriške somiče (*Ameiurus melas*) smo pripeljali iz ribnikov ribogojnice v Pernici pri Mariboru. V akvarijski sobi Oddelka za biologijo smo jih v skupinah po 100 namestili v 500 litrske prezračevane kadi. Somiče smo zdravili proti parazitom in bakterijam s 0,4% raztopino kuhinjske soli in malahitnim zelenilom. Vzdrževali smo jih na 12/12 urnem dnevno nočnem ritmu. Vsaj 2 meseca pred vedenjskimi poizkusi smo posamične somiče prestavili v prezračevane 60 litrske (48 cm×38 cm×33 cm) akvarije. Somiči so si v tem času ustvarili koncept eksperimentalnega akvarija in so pričeli redno jesti. Prehrana je vsebovala koščke osličevega mesa.

3.2 IZREZANJE VOHALNIH ROZET

Somiče smo anestezirali v vodni raztopini MS 222 (etil-3-aminobenzoat metan-sulfonat, 1:8000, Fluka) in jih z bucikami pritrdili na vosek. Med operacijo je škrge somičev oblivala voda z anestetikom. Da bi preprečili izsuševanje kože smo telo somiča ovili v vlažne bombažne robčke. Pod stereomikroskopom (SMZ-1, Nikon) smo kirurško odstranili nosni most in izpostavili vohalni epitel. Somičem smo izrezali celoten ali večji del vohalnega epitela. Delno izrezani vohalni organi so vsebovali majhne dele dvanajstih do osemnajstih (slika 2a) ali le treh do sedmih (slika2b) vohalnih lamel in rafo. Po operaciji smo somiče potopili v prezračevano vodovodno vodo in ko so pričeli samostojno dihati smo jih prestavili v 60 litrske akvarije. Med regeneracijo vohalnega organa smo somiče redno hranili.

3.3 FOTOGRAFIRANJE VOHALNIH ORGANOV

Anestezirane somiče (MS 222; 1:8000) smo namestili v plastično posodo, napolnjeno z vodo, ki je vsebovala anestetik. Ribo smo z bucikami pritrdili na leseno dno plastične posode in z digitalnim fotoaparatom Coolpix 4500 (Nikon) posneli sprednji zgornji del glave. Fotografije regeneriranih vohalnih organov smo posneli pod stereomikroskopom (Leica) 2, 4 in 18 mesecev po začetku regeneracije vohalnega organa.

3.4 VRSTIČNA ELEKTRONSKA MIKROSKOPIJA (PRILOGA A)

Ameriške somiče z intaktnimi in regeneriranimi vohalnimi organi smo anestezirali z vodno raztopino MS 222 (1:5000) in jih z bucikami pritrdili na dno preparacijske posode. S perfuzijo z ribjim Ringerjem skozi srce smo somičem iz žil izpodrinili kri. Izolirali smo vohalne organe in jih izprali z 0.1 M kakodilatnim pufrom. Izolirana tkiva smo fiksirali v raztopini 1 % glutaraldehida in 0.5 % formaldehida v 0.1 M kakodilatnem pufru. Vohalne organe smo kontrastirali z osmijevim tetraoksidom (OsO_4), dehidrirali v naraščajoči lestvici vodnih raztopin alkohola in acetona, ter jih posušili v ogljikovem dioksidu (CO_2) pri kritični točki. Iz posušenih intaktnih in regeneriranih vohalnih organov smo izolirali po nekaj vohalnih lamel in jih naparili z zlatom. Vzorce smo si ogledali in slikali pod vrstičnim elektronskim mikroskopom (Jeol 840a).

3.5 BARVANJE Z DiI

Anestezirane somiče (MS 222; 1:5000) smo z bucikami pritrdili na vosek preparacijske posode. Somičem smo s perfuzijo preko srca dovajali raztopino ribjega Ringerja, temu je sledil 4 % formaldehid v 0.1 M fosfatnem pufru. Raztopino formaldehida smo pripravili iz paraformaldehida (Merck) nekaj ur pred perfuzijo. Med

fiksacijo smo nosno votlino občasno spirali z mešanico 0.2 % glutaraldehida in 4 % formaldehida v pufru. Izolirali smo anteriorni dorzalni del glave somiča in izpostavili eno od območij: anteriorno dorzalno, lateralno dorzalno, mediano dorzalno, posteriorno dorzalno, anteriorno ventralno, lateralno ventralno, mediano ventralno oziroma posteriorno ventralno območje vohalnega bulbusa. Vzorce, ki so vsebovali izoliran del glave somiča, smo pustili čez noč v 4 % formaldehidu v pufru. Naslednji dan smo s pomočjo entomološke igle vstavili majhne kristale barvila DiI (1, 1`-dioktadecil-3, 3, 3`, 3`-tetrametilindo-karbocianin, Molecular probes) v izpostavljeno območje vohalnega bulbusa. Vohalni bulbus smo prekrili z 2 % agarjem in s tem preprečili prenašanje kristalov barvila. Med 19-25 dnevno inkubacijo smo vzorce tkiva hranili v 4 % nevtraliziranem formaldehidu na sobni temperaturi v temi. Po koncu inkubacije smo izolirali vohalni epitel z vohalnim bulbusom in vzorce vklopili v 15 % želatino. Želatinske bloke smo čez noč fiksirali v 4 % formaldehidu v pufru. Naslednji dan smo na vibratomu (Vibratome 1000 Plus, The Vibratome Company, St. Luis) narezali 50 µm debele rezine in jih z mikroskopom (Axioscope, Opton, West Germany) opazovali pod epifluorescenco. Fotografije intaktnih in regeneriranih vohalnih epitelov smo posneli z digitalnim fotoaparatom Coolpix 4500 (Nikon).

3.6 MERJENJE ELEKTROOLFAKTOGRAMA (EOG)

Ameriške somiče smo anestezirali v vodni raztopini MS 222 (1:8000) in jih prenesli v poskusno posodo. Glavo somiča smo vpeli v plastično sponko. Med snemanjem je škrge somiča oblivala voda z anestetikom. Podobno kot pri podvodnih EOG meritvah (Silver s sod., 1976) je vodni tok s hitrostjo ~18 ml/min preko Y-cevke vstopal v dražilno in uravnalno cevko. Cevki sta se preko T ventila ponovno združili v enotno izvodno cevko (0.8 mm notranji premer). Prosti konec izvodne cevke smo s pomočjo mikromanipulatorja (Narishige MM-33N) namestili na rob vohalne odprtine in ga usmerili proti regeneriranim vohalnim lamelam. Z brizgo smo preko T-cevke v dražilno cevko dovedli 0.5 ml 10^{-4} M (10^{-2} M) vodne raztopine aminokislin (L-prolina). Obrnili smo T ventilček in s tem vodni tok preusmerili iz uravnalne v dražilno cevko. V 1-3 sekundah je dražljaj dosegel regeneriran vohalni organ (Koce,

1999). Čas med zaporednimi draženji je bil vsaj 1.5 min. EOG signal smo snemali preko $Ag/AgCl_2$ elektrode z agarjevim mostičkom, ki je bila nameščena v vodi ~1mm nad regeneriranim vohalnim organom. Signal smo 100 × ojačili (Grass P18D ojačevalec) in z risalnikom (OmniScribe recorder; Industrial Scientific, TX, ZDA) zapisali na milimetrski papir. Z ravnilom smo na milimetrskem papirju izmerili največjo amplitudo EOG odziva [mm].

3.7 ANALIZA EOG MERITEV

Amplitude EOG odzivov različnih aminokislin smo standardizirali z amplitudo EOG odziva na 10^{-4} M L-alanin. S Pearsonovim korelacijskim koeficientom [R] smo primerjali relativne EOG amplitude različnih aminokislin v intaktnih (Koce, 1997) in regeneriranih vohalnih organih.

3.8 VEDENJSKI TESTI

Pri somičih, ki smo jim pustili majhne dele treh do sedmih vohalnih lamel in rafo, smo začeli z vedenjskimi testi šest mesecev po izrezanju rozet. Z L-norvalinom (L-nVal) smo somiče dražili 1-5 × dnevno. Devetdestet do stodvajset sekund po vbrizganju L-nVal so somiči prejeli nagrado, 1-3 koščke osličevega mesa. Ozmične ribe smo lahko z nagrado pogojili, anozmični somiči pa zaradi nagrade niso spremenili jakosti odziva. Vohalno razlikovanje aminokislin smo ugotavljali 1-14 mesecev po začetku postopka draženja z L-nVal. Vodne raztopine aminokislin ($3*10^{-2}$ M) smo pripravili manj kot 30 minut pred testiranjem. Dva mililitra dražljaja smo vbrizgali v vodo akvarija na področju, ki so ga mešali mehurčki zraka. Ti so prožili turbulentne vodne tokove, ki so povzročali navpično mešanje vodne mase. Migotice z visokimi koncentracijami dražljaja so tako dosegle dno akvarija. Dražljaj je somiča dosegel v manj kot 30 sekundah (Valentinčič in Caprio, 1994). Med 90 sekundnim testom smo ocenjevali aktivnost somiča s štetjem obratov nad 90º. V kontrolnih testih smo v akvarij vbrizgali

vodovodno vodo. Vse poskuse smo snemali z digitalno video kamero (MV1, Canon). Statistično (Wilcoxonov test predznačenih rangov; $p<0.05$) smo primerjali število obratov, ki jih je somič naredil po vbrizganju ponavljajoče predstavljene in testne aminokisline. Pri ozmičnih somičih so testi razlikovanja aminokislin potekali 3-5 krat dnevno. Somiče smo izmenjujoče dražili s pogojno in nepogojnimi aminokislinami. Anozmične somiče smo dnevno dražili le z dvema različnima aminokislinama. Prva predstavljena aminokislina je bila, od dveh predstavljenih aminokislin, vedno manj učinkovit okušalni dražljaj (Caprio s sod., 1975). Tako smo preprečili navzkrižno adaptacijo okušalnih dražljajev, zaradi katere bi se verjetnost odziva somičev na okušalni dražljaj močno znižala.

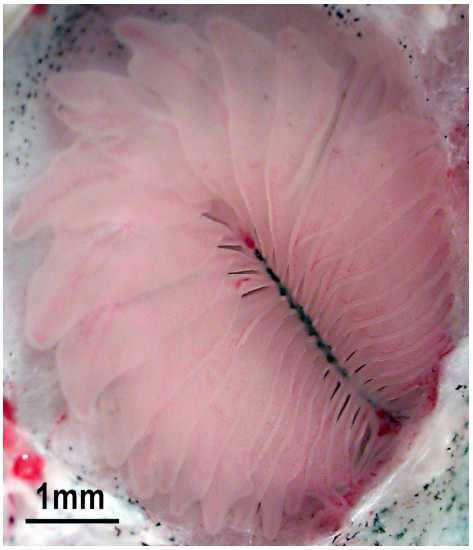
3.9 AMINOKISLINE UPORABLJENE V EOG MERITVAH IN VEDENJSKIH TESTIH

Aminokisline (čistost ≥ 98%) L-norvalin, L-arginin hidroklorid (L-Arg), (L-nVal), L-norlevcin (L-nLeu), L-valin (L-Val), L-cistein hidroklorid (L-Cys), L-metionin (L-Met), L-lizin hidroklorid (L-Lys) so bile izdelki Sigme (Sigma Chemical, St.Luis, MO), aminokisline L-prolin (L-Pro), L-alanin (L-Ala) in L-levcin (L-Leu) pa izdelki Fluke (Fluka Chemica-Biochemica, Switzerland).

4 REZULTATI

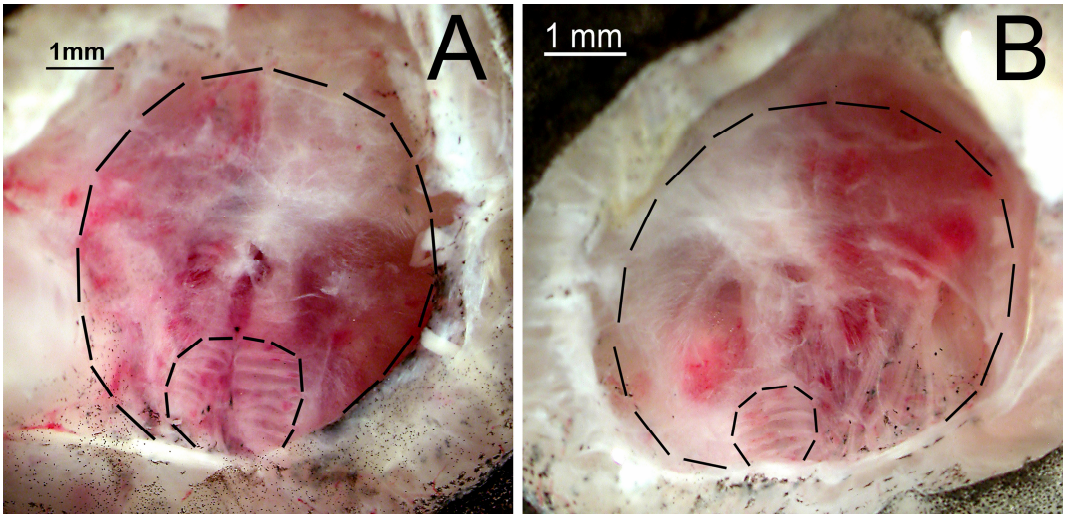
4.1 OBSEG REGENERACIJE VOHALNEGA ORGANA

Vohalne rozete ameriških somičev (*Ameiurus melas*) vsebujejo 24-34 listastih vohalnih lamel, ki so pritrjene na osrednjo rafo (Slika 1). Po delni odstranitvi vohalnih rozet so vohalni organi regenerirali do različnih velikosti in oblik; od majhnih popolnih vohalnih rozet (Slika 4), pahljačastih rozet (Slika 5) do nekaj posameznih lamel (Slika 9). Obseg regeneracije vohalnega organa je bil odvisen od površine vohalnega epitela, ki smo ga po operaciji pustili tik ob rafi. Majhne popolne vohalne rozete (Slika 4) in vohalne rozete pahljačastih oblik (Slika 5) so zrasle v primeru, ko smo med kirurško odstranitvijo vohalnega organa pustili majhne dele dvanajstih do osemnajstih vohalnih lamel tik ob rafi (N= 30; Slika 2A). Po obsežni odstranitvi vohalnega organa smo tik ob rafi pustili majhne dele treh do sedmih vohalnih lamel (N=26; Slika 2B). Po dveh mesecih so regenerirale majhne deformirane vohalne rozete z nekaj vohalnimi lamelami (Slika 8); pogosto se je na mestu prvotne vohalne rozete oblikovalo vezivno in epitelno tkivo, ki ni imelo oblike rozet (Slika 10 in 11). Po popolni odstranitvi vohalnih rozet (N=36) je pri mladih somičih (< 2 leti) kirurško rano zarasla koža (N=20; Slika, 3A), pri starih somičih (> 3 leta) sta mesto prvotne vohalne rozete prekrila vezivo in tanka plast epitela (N=16; Slika 3B).



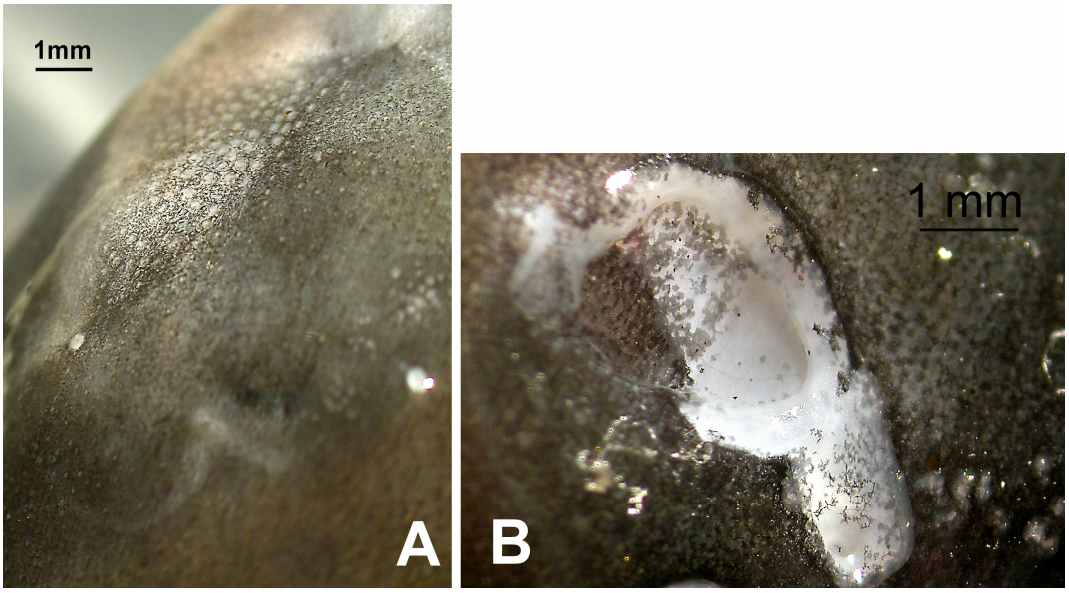
Slika 1: Intaktna vohalna rozeta ameriškega somiča z 28 listastimi vohalnimi lamelami, ki so nameščene na osrednjo rafo.

(Fig. 1 Intact olfactory roseta of black bullhead catfish with 28 leaf-like olfactory lam ellae attached to the middle rapha.)



Slika 2: Majhni deli dvanajstih (**A**) in majhni deli štirih (**B**) vohalnih lamel z rafo, ki smo jih po delni odstranitvi vohalnih rozet somiča pustili v vohalni votlini. Dolge črtkane črte označujejo obseg intaktne vohalne rozete, medtem ko kratke črtkane črte obkrožajo ostanek vohalnega epitela.

(Fig. 2 Partial excision of catfish olfactory rosetae left small portions of twelve (**A**) or four (**B**) olfactory lamellae with rapha in the olfactory cavity. Long dashed lines mark position of intact olfactory roseta, whereas short dashed lines outline the remaining olfactory epithelium.)

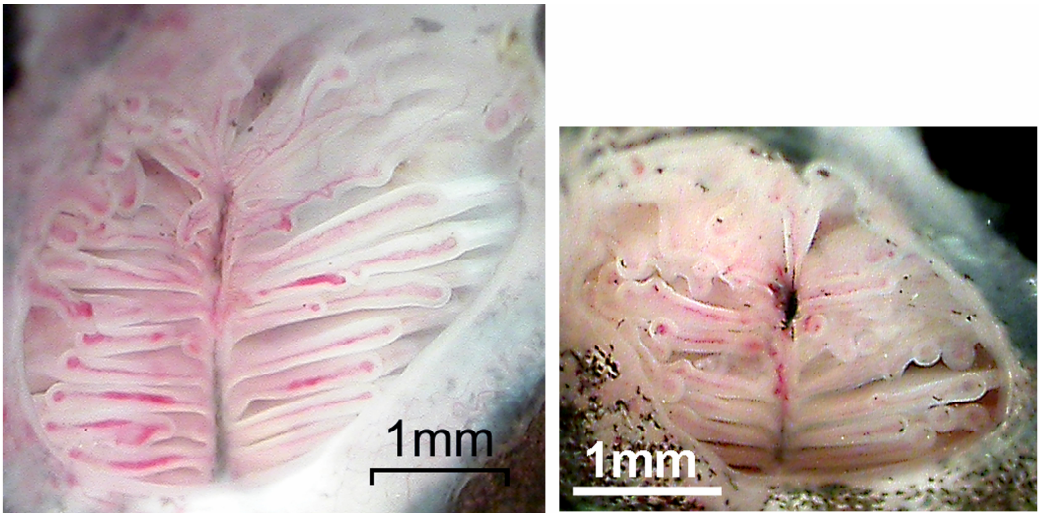


Slika 3: Vohalna votlina zaraščena s kožo pri >dve leti starem somiču (**A**) ter vezivo in tanek epitel pri < tri leta starem somiču (**B**).

(Fig. 3 Olfactory cavity covered with skin in >2 years old catfish (**A**) and connective and epithelial tissue in <3 years old catfish (**B**).)

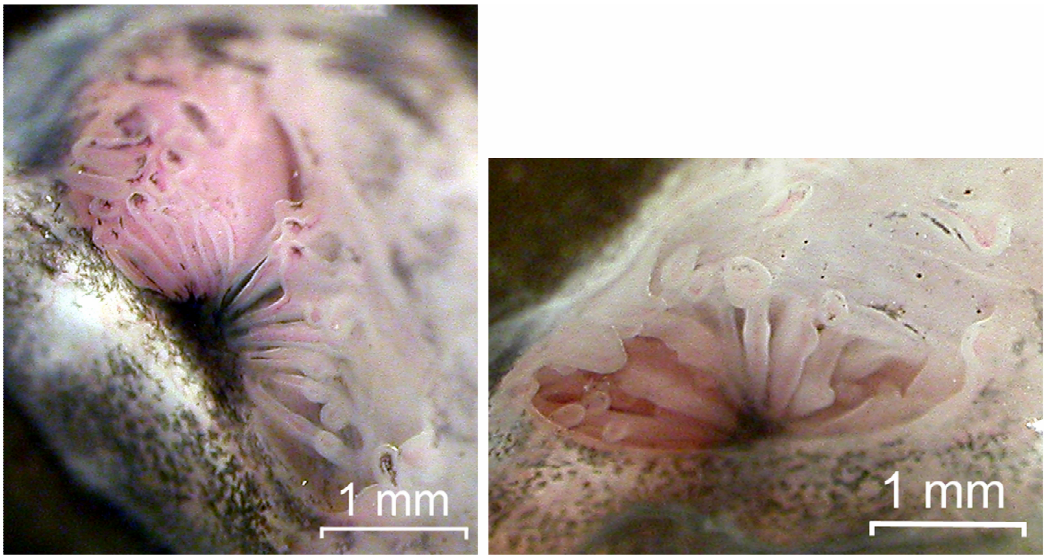
4.2 REGENERIRANE VOHALNE ROZETE

Dva meseca po delni odstranitvi vohalnih rozet so, iz majhnih delov dvanajstih do osemnajstih vohalnih lamel z rafo (Slika 2A) regenerirale majhne popolne ali pahljačaste vohalne rozete, ki so bile znatno manjše od intaktnih vohalnih rozet (Slika 4 in 5). Posamezna regenerirana rozeta je vsebovala 13-22 vohalnih lamel. Podobno kot v intaktnih lamelah (6A) so se v regeneriranih lamelah vohalne celice nahajale v čutilnem epitelu ob osrednji rafi (slika 6B,C). Ostale predele vohalnih lamel je prekrival nečutilni kinociliarni epitel (slika 6B). Nekatere od regeneriranih vohalnih lamel so bile prstastih oblik (Slika 4 in 5). Prstasti izrastki so bili pogosto nameščeni na robu regenerirane rozete (Slika 4 in 5).



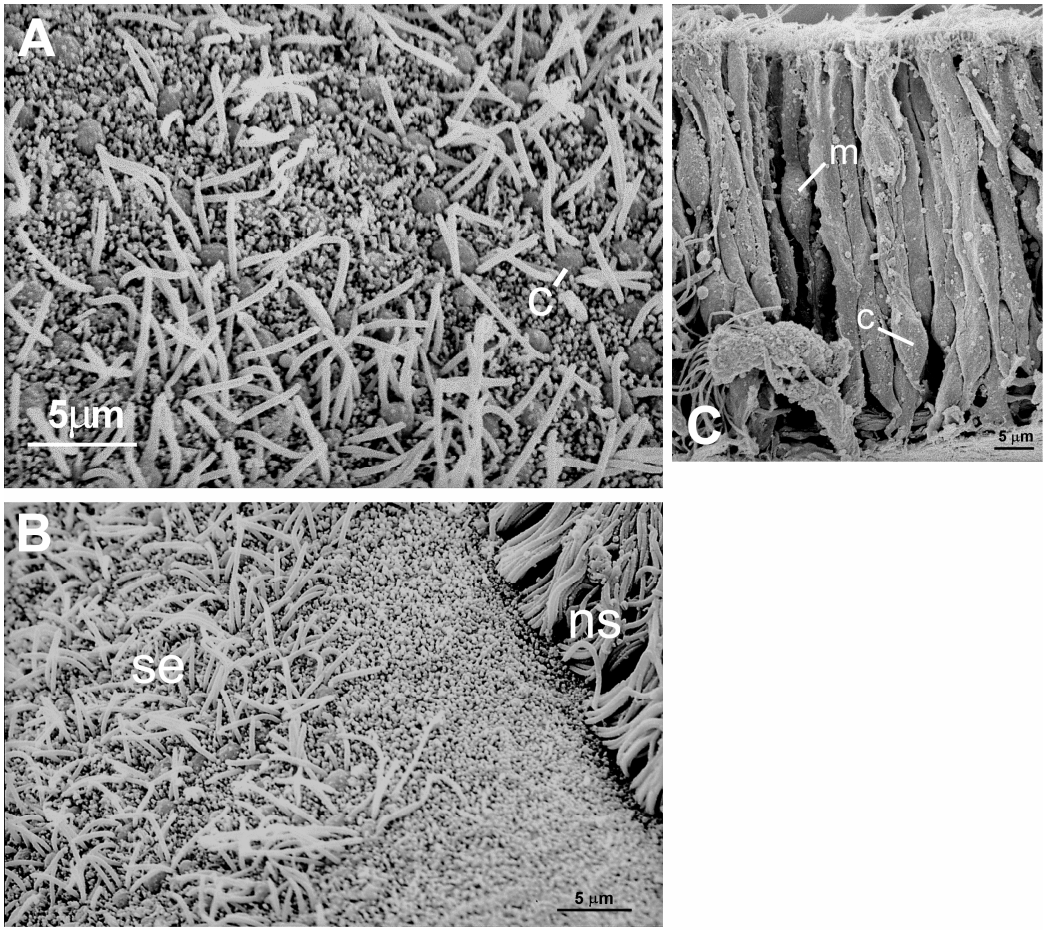
Slika 4: Regenerirani vohalni rozeti ameriškega somiča. Večina vohalnih lamel je listastih, nekaj pa prstastih. Prstasti izrastki lamel so nameščeni na robu rozet.

(Fig. 4 Regenerated olfactory rosetae of black bullhead catfish. Olfactory lamellae are leaf-like; some lamellae are finger-like. Finger-like emergences are also located at the edge of roseta.)



Slika 5: Pahljačasti regenerirani vohalni rozeti. Listaste vohalne lamele so pripete na kratko osrednjo rafo. Nekatere lamele so prstastih oblik. Prstasti izrastki so nameščeni na robu rozet.

(Fig. 5 Fan-like regenerated olfactory rosetae. Leaf-like lamellae are attached to short rapha. Some of lamellae are finger-like. Finger-like emergences are located at the edge of rosetae.)



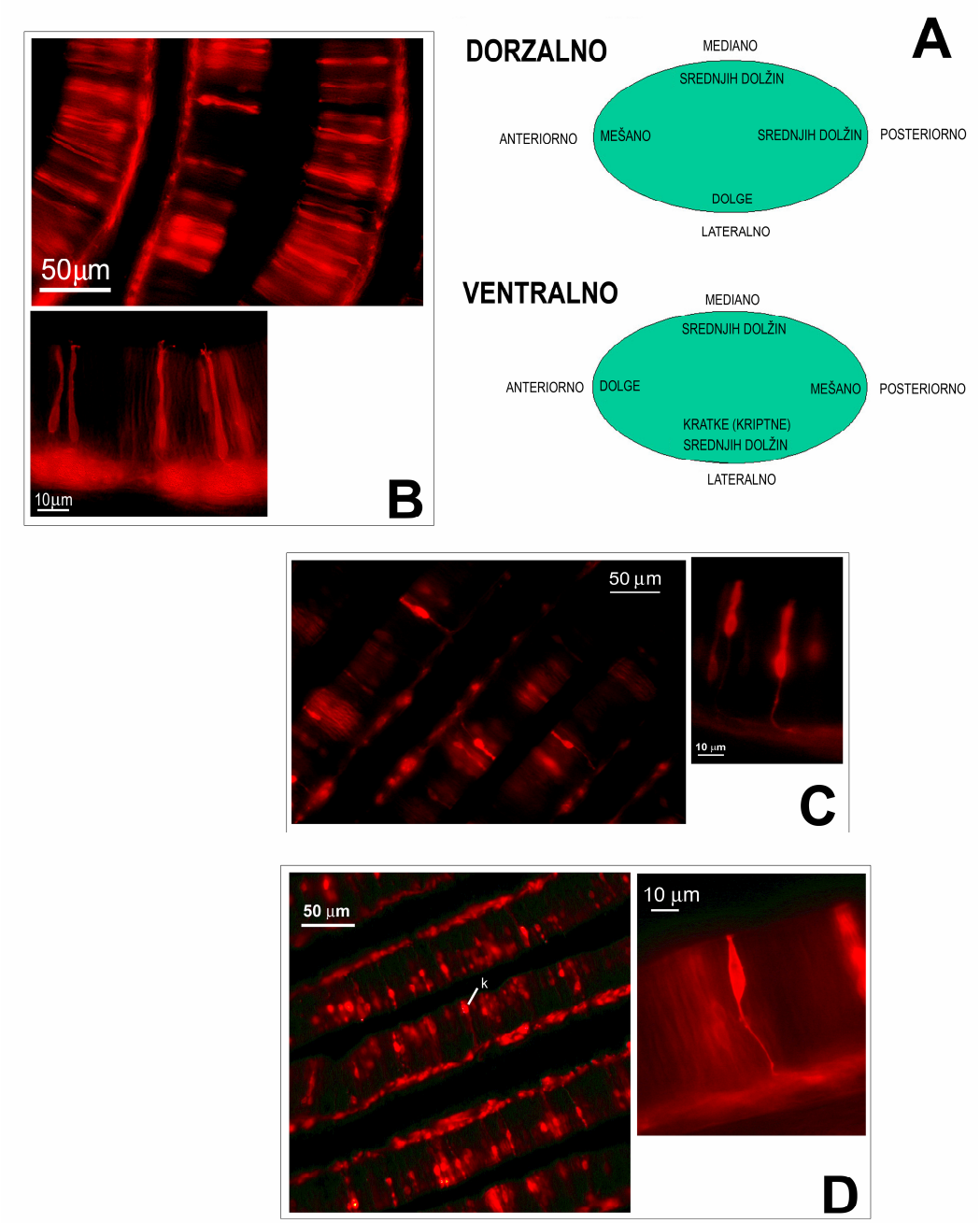
Slika 6: Posnetki intaktnega (**A**) in regeneriranega (**B** in **C**) vohalnega epitela ameriškega somiča z vrstičnim elektronskim mikroskopom. Regenerirani vohalni epitel (se) vsebuje dolge vohalne celice s cilijami (c) in vohalne celice srednjih dolžin z mikrovili (m) (**C**). Nečutilni epitel (ns) sestavljajo celice s kinocilijami (**B**).

(Fig. 6 Intact (**A**) and regenerated (**B** and **C**) olfactory epithelium of black bullhead catfish. Regenerated sensory epithelium (se) contains tall ciliated cells (c) and intermediate microvillar cells (m) (**C**). Non-sensory epithelium (ns) consists of kino-ciliated cells (**B**).)

4.2.1 Živčne povezave med regeneriranimi vohalnimi rozetami in vohalnim bulbusom

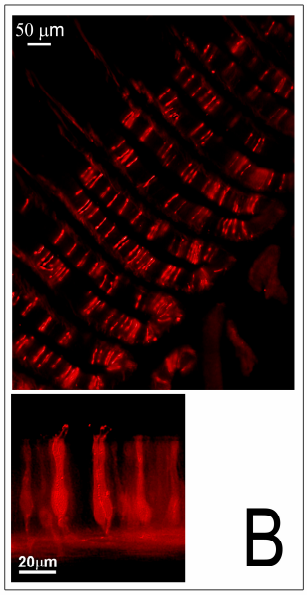
Retrogradno iz vohalnega bulbusa smo, z uporabo barvila DiI, v vohalnem epitelu somičev obarvali različne tipe vohalnih celic (intaktni vohalni organi, N=52; Slika 7; regenerirani vohalni organi, N=17; Slika 8). V intaktnih vohalnih organih so se dolge vohalne celice s cilijami (Slike 7A, B) obarvale po vnosu DiI kristalov v anteriorno ventralno (N=18), posteriorno ventralno (N=4), anteriorno dorzalno (N=5) in lateralno dorzalno (N=2) območje vohalnega bulbusa. Vohalne celice srednjih dolžin z mikrovili (Slika 7A, C) so se obarvale po vnosu DiI kristalov v mediano ventralno (N=3), lateralno ventralno (N=15), mediano dorzalno (N=2) in posteriorno dorzalno (N=3) območje vohalnega bulbusa. Kratke, kriptne celice (Slika 7A, D) smo opazili le v dveh vohalnih organih, po vsaditvi DiI kristalov v lateralno ventralno območje vohalnega bulbusa. Telesa kriptnih celic so ovalna, nameščena blizu površine čutilnega epitela (Slika 7D). Telesa vohalnih celic s cilijami so stekleničastih oblik, segajo so od površine čutilnega epitela do bazalne lamine (Slike 7B). Telesa srednje dolgih vohalnih celic z mikrovili ne segajo do bazalne lamine, zavzemajo drugo tretjino debeline čutilnega epitela (Slika 7C).

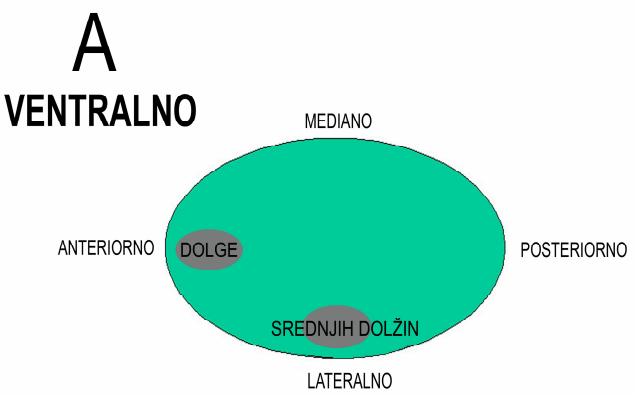
V regeneriranih vohalnih rozetah (Slika 8) so se, prav tako kot v intaktnih vohalnih organih (Slika 7), dolge vohalne celice (Slika 8A, B) obarvale po vnosu DiI kristalov v anteriorno ventralno območje (N=9), medtem ko so se vohalne celice srednjih dolžin (Slika 8A, C) obarvale po vnosu DiI kristalov v lateralno ventralno območje vohalnega bulbusa (N=8).

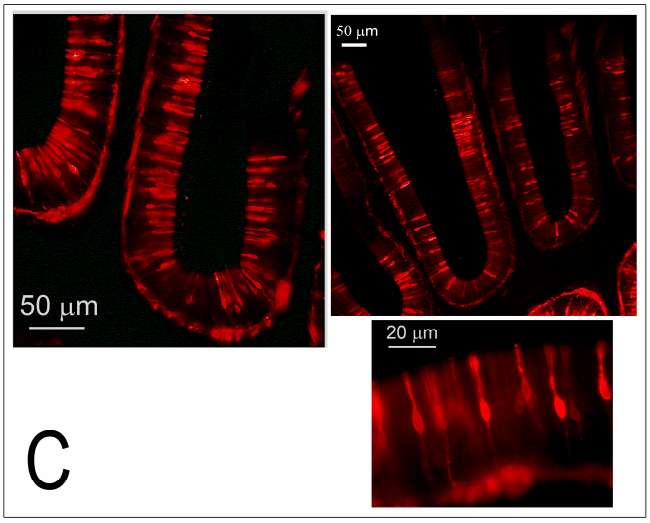


Slika 7**:** DiI kristale smo vnesli v označena področja vohalnega bulbusa (**A**). V vohalnem epitelu so se obarvale dolge vohalne celice s cilijami (**B**), vohalne celice srednjih dolžin z mikrovili (**C**) in kratke kriptne celice (**D,** k).

(Fig 7 DiI crystals were inserted at indicated areas of olfactory bulb (**A**). In the olfactory epithelium tall ciliated ORNs (**B**), intermediate microvillous ORNs (**C**) and short crypt cells (**D,** k) were labeled.)





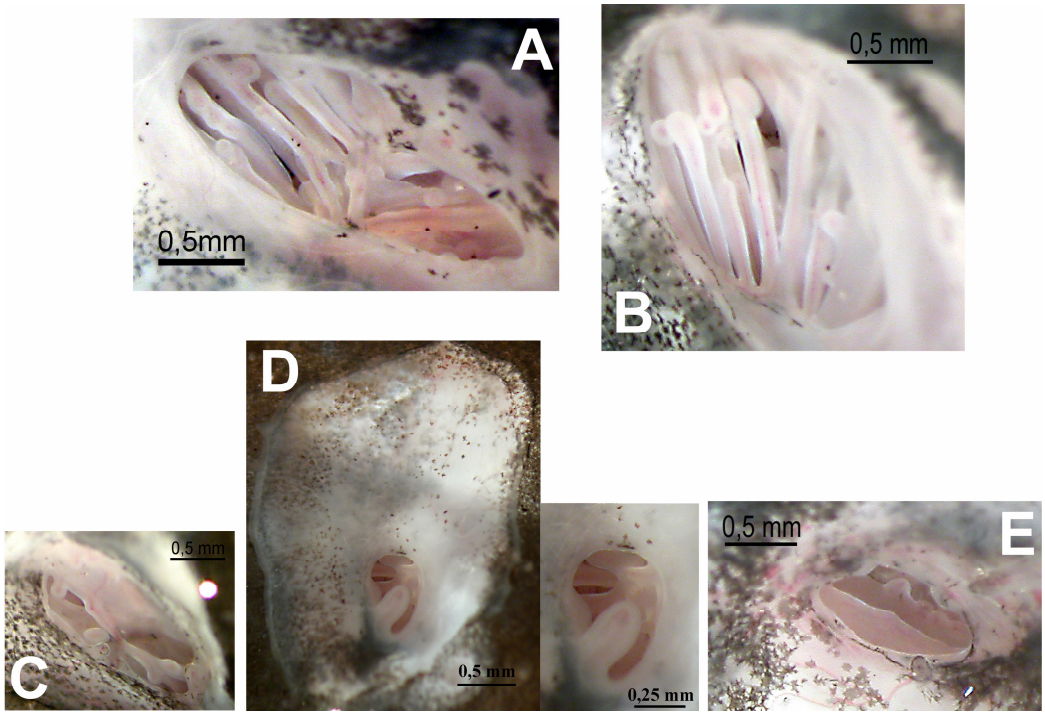


Slika 8: V regeneriranih vohalnih rozetah smo DiI kristale vnesli v sivo označena območja vohalnega bulbusa (**A**). V vohalnem epitelu so se obarvale dolge vohalne celice (**B**) in vohalne celice srednjih dolžin (**C**).

(Fig 8 In regenerated olfactory rosetae we inserted DiI crystals into gray marked OB areas (**A**). In olfactory epithelium tall (**B**) and intermediate (**C**) olfactory receptor neurons were labeled.)

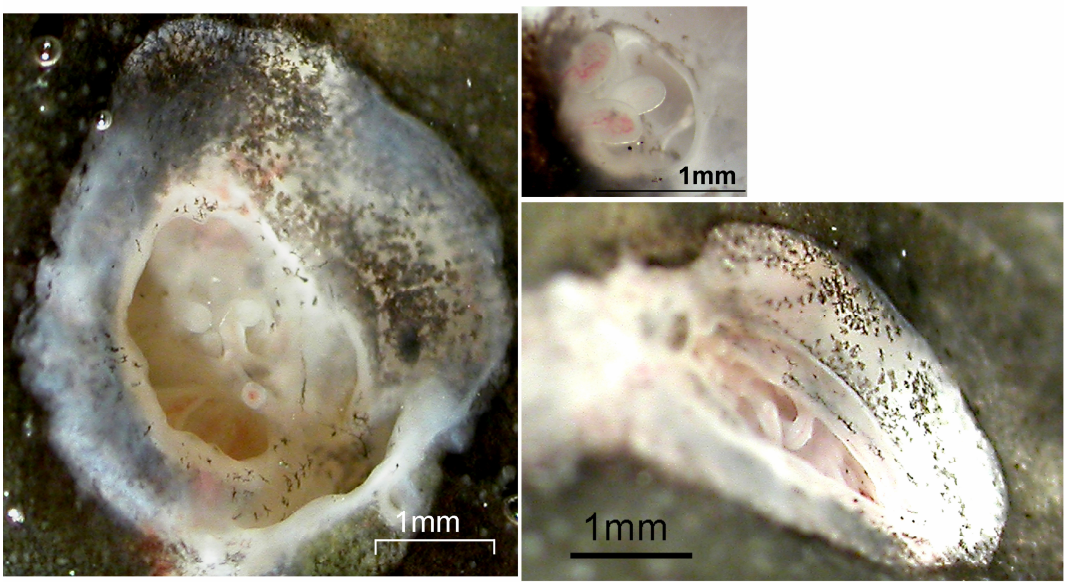
4.3 MAJHNI REGENERIRANI VOHALNI ORGANI

Dva meseca po obsežni odstranitvi vohalnih rozet, so iz majhnih delov treh do sedmih vohalnih lamel in rafe (Slika 2B) regenerirale deformirane vohalne rozete z le nekaj majhnimi vohalnimi lamelami (N=5; Slika 9); pogostokrat so na mestu prvotne vohalne rozete zrasla tkiva v obliki 1-9 prstastih lamel (N=8; Slika 10) ali v obliki nepravilnih izrastkov iz epitela in veziva (N=13; Slika 11). Deformirane vohalne rozete so vsebovale 2, 4, 5, 7 ali 12 majhnih vohalnih lamel (Slika 9). Najmanjši vohalni organ sta gradili dve listasti vohalni lameli (Slika 9E). Ostali vohalni organi so vsebovali listaste in nekaj prstastih lamel (Slika 9A, B, C in D).



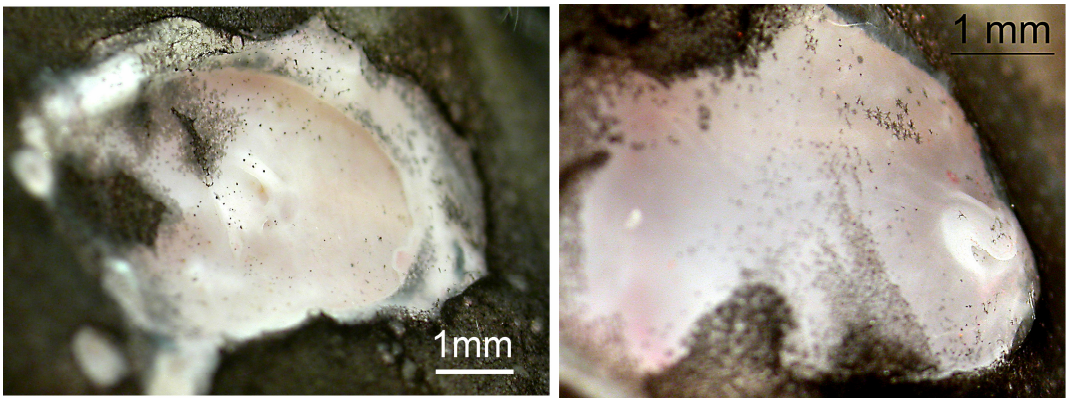
Slika 9: Regenerirane deformirane vohalne rozete somičev z 2 (**E**), 4 (**D**), 5 (**C**), 7 (**B**) in 12 (**A**) vohalnimi lamelami.

(Fig 9 Regenerated deformed olfactory rosetae of catfish with 2 (**E**), 4 (**D**), 5 (**C**), 7 (**B**) and 12 (**A**) olfactory lamellae.)



Slika 10: Regenerirane prstaste lamele v nekdanji vohalni votlini somiča.

(Fig 10 Regenerated finger-like lamellae in catfish olfactory cavity.)

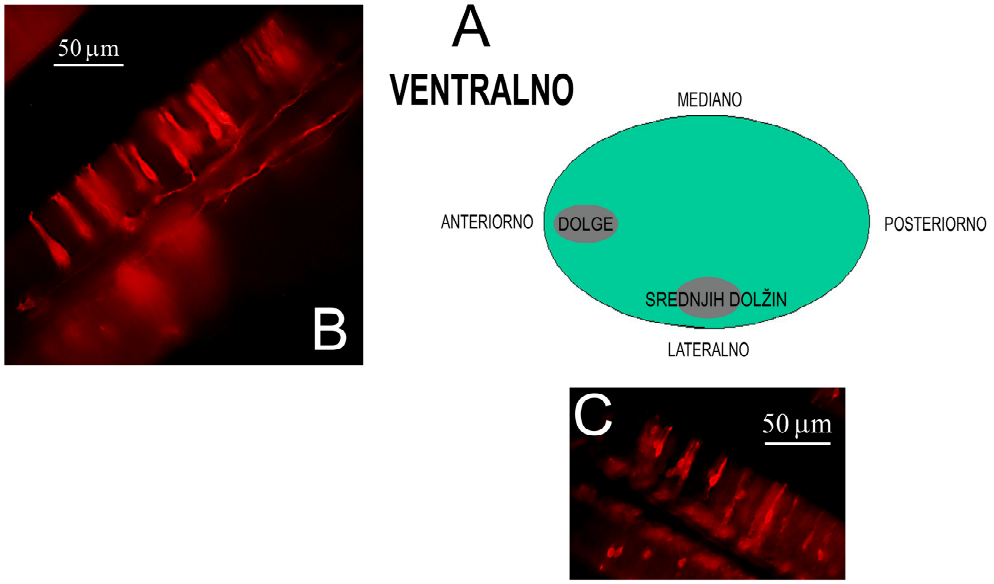


Slika 11: Neoblikovana epitelna in vezivna tkiva v nekdanji nosni votlini somiča.

(Fig 11 Epithelial and connective tissues without special shape in catfish olfactory cavity.)

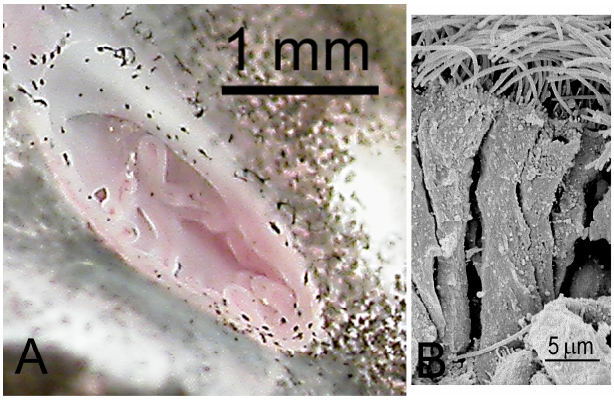
4.3.1 Živčne povezave med majhnimi regeneriranimi vohalnim lamelami in vohalnim bulbusom

Opazovali smo živčne povezave med regeneriranim vohalnim epitelom in vohalnim bulbusom obarvane z barvilom DiI. Enako kot v intaktnih vohalnih organih (Slika 7A in B) so se v majhnih regeneriranih vohalnih organih s štirimi (Slika 9D) ali dvanajstimi vohalnimi lamelami (Slika 9A), po vstavitvi DiI kristalov v anteriorno ventralno območje vohalnega bulbusa, obarvale dolge vohalne celice s cilijami (Slika 12 A in B). V najmanjšem vohalnem organu z le dvema lamelama (Slika 9E) smo DiI kristale vsadili v lateralno ventralno območje vohalnega bulbusa (Slika 12A). V regeneriranem vohalnem epitelu so se, podobno kot v intaktnem (Slika 7A in C), v večini primerov obarvale celice srednjih dolžin z mikrovili (Slika 12C). V prstastih lamelah nismo opazili živčnih povezav med epitelom in vohalnih bulbusom, pokrival jih je izključno kinociliarni nečutilni epitel (N=2; Slika 13).



Slika 12 V majhnih regeneriranih vohalnih organih smo vnesli DiI kristale v sivo označena območja vohalnega bulbusa (**A**). V vohalnem epitelu so se obarvale dolge vohalne celice (**B**) in vohalne celice srednjih dolžin (**C**).

(Fig. 12 In small regenerated olfactory organs we inserted DiI crystals into gray marked OB areas (**A**). In olfactory epithelium tall ciliated (**B**) and intermediate microvillar olfactory receptor neurons were labeled (**C**).)

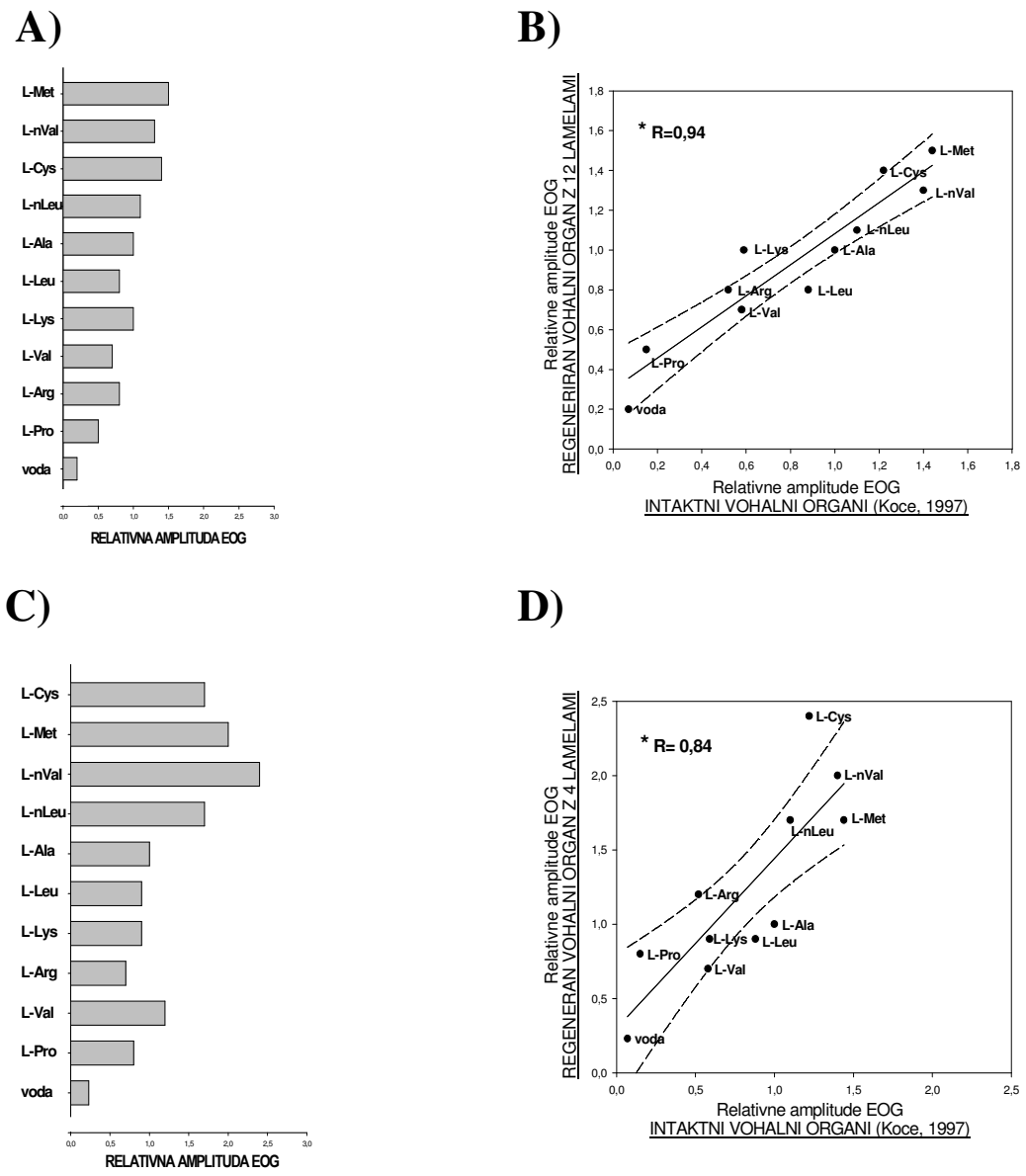


Slika 13: Prstaste regenerirane lamele (**A**), ki jih pokriva epitel s kinocilijami (vrstični elektronski mikroskop) (**B**).
(Fig. 13 Regenerated finger-like lamellae (**A**) are covered by kino-ciliated cells (**B**) (scanning electron microscope).)

4.3.2 Elektroolfaktogram (EOG) majhnih regeneriranih vohalnih organov

Deformirane vohalne rozete z nekaj vohalnimi lamelami (N=5; Slika 9) so se 18 mesecev po obsežni odstranitvi vohalnih rozet odzivale na draženje z aminokislinami z razmeroma veliko amplitudo EOG [~10mV; intaktni ~30mV]. V intaktnih (Koce, 1997) in regeneriranih vohalnih organih so visoko učinkovite aminokisline (L-Cys in L-Met) prožile velike amplitude EOG, nizko učinkovite aminokisline (L-Val in L-Pro) pa majhne amplitude EOG (Slika 14A,C in 16C). Relativne (relativne na L-Ala) amplitude EOG različnih aminokislin v intaktnih in regeneriranih vohalnih organih smo primerjali s Pearsonovim korelacijskim koeficientom (R; Slika 14B, D in 16C), korelacije so bile visoke in statistično značilne (R= 0.79-0.94; $p<0.05$). Najmanjši vohalni organ z dvema vohalnima lamelama je imel, v primerjavi z intaktnimi vohalnimi organi, najnižjo korelacijo (R= 0,79; Slika 16C), medtem ko smo največjo korelacijo izračunali za največji regeneriran vohalni organ z 12 lamelami (R= 0,94; Slika 14B).

V tkivih, ki so jih gradile bodisi prstaste lamele bodisi nepravilni izrastki iz epitela in veziva (Slika 10 in 11) nismo zaznali elektrofizioloških odzivov na aminokisline.

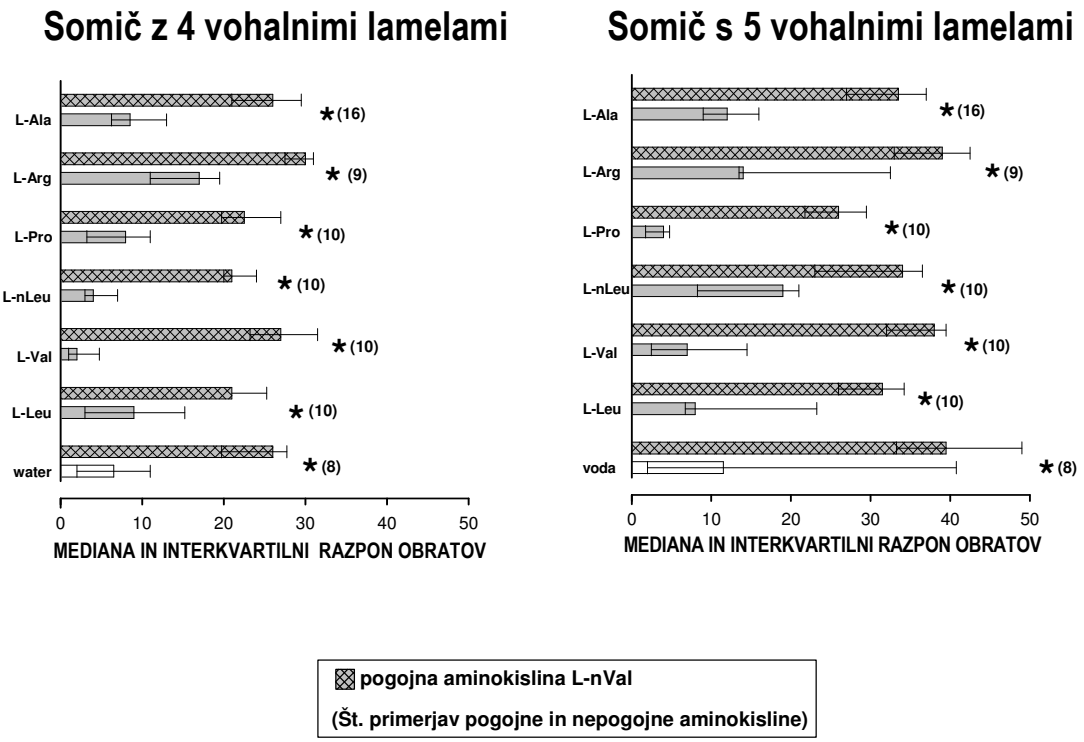


Slika 14: Relativne (rel. L-Ala) EOG amplitude po draženju majhnih regeneriranih rozet z dvanajstimi (**A**) oziroma štirimi (**C**) lamelami z amino kislinami. R = Pearsonov korelacijski koeficient med relativnimi EOG amplitudami pri intaktnih in majhnih regeneriranih rozetah (**B, D**). Zvezdice označujejo značilne korelacije [$p<0.05$].

(Fig. 14 Relative (L-Ala) EOG amplitudes of small regenerated olfactory organs with twelve (**A**) and five (**C**) lamellae after amino acid stimulation. R = Pearson correlation coefficient between EOG amplitudes for intact and regenerated olfactory organs (**B, D**); stars indicate significant correlation [$p<0.05$].)

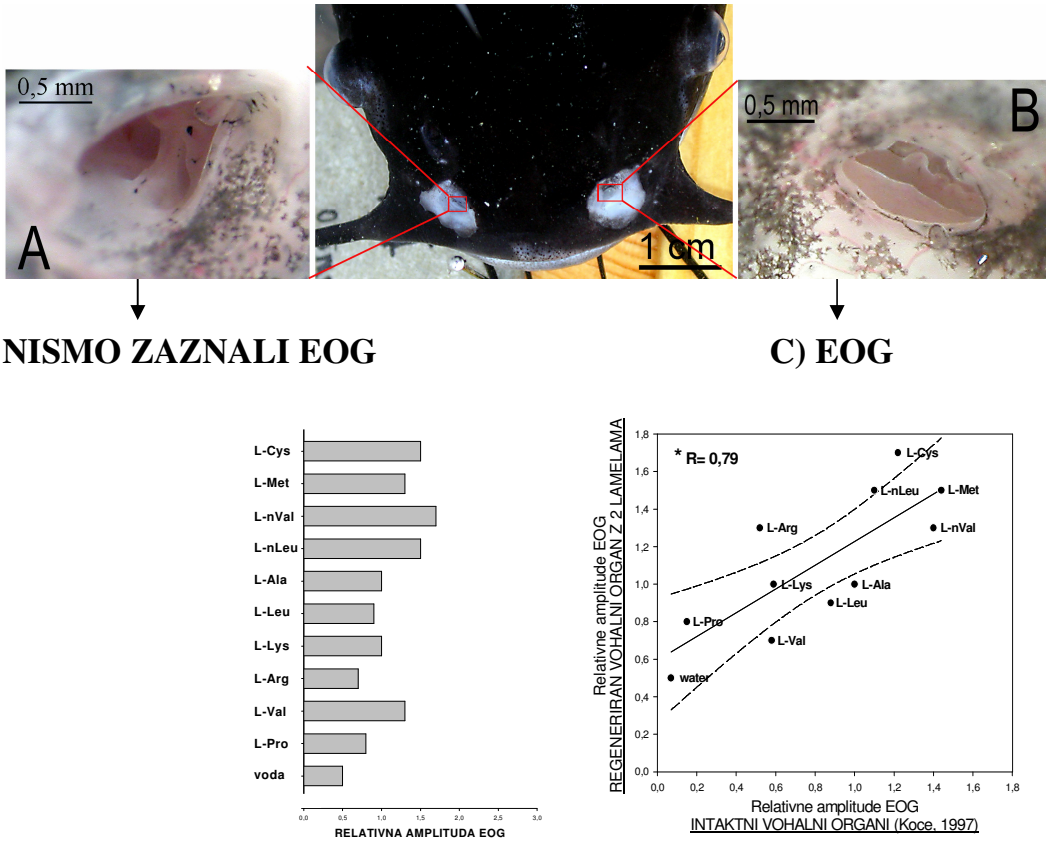
4.3.3 Razlikovanje aminokislin pri somičih z majhnimi regeneriranimi vohalnimi organi

S pogojevanjem ameriških somičev (N=13) na L-nVal smo pričeli 6 mesecev po delni odstranitvi vohalnih rozet in nadaljevali do 20 meseca po operaciji. Po ~ 30 pogojevanjih smo somiče testirali za vohalno razlikovanje aminokislin. Prvi somič je začel razlikovati pogojno od nepogojnih aminokislin 8 mesecev po operaciji (Slika 15levo). Odziv prvega somiča na pogojno aminokislino L-nVal je bil vsaj dvakrat večji kot odziv na ostale aminokisline. Dvanajst mesecev po operaciji so štiri ribe od trinajstih razlikovale L-nVal od ostalih aminokislin. Somič z najmanjšim vohalnim organom (Slika 16), ki je vseboval le dve listasti vohalni lameli (Slika 16B), je začel razlikovati aminokisline dvajset mesecev po operaciji (Slika 16D). Odziv tega somiča na pogojno aminokislino L-nVal je bil že v trenutku začetka razlikovanja dvakrat večji kot odziv somiča na nepogojno aminokislino L-Ala. Vsi somiči, ki so razlikovali aminokisline, so imeli samo po en delujoč vohalni organ. Na pogojno aminokislino L-nVal se je vsak od ozmičnih odzval vsaj dvakrat bolj intenzivno, kot na nepogojne aminokisline (Slika 15).

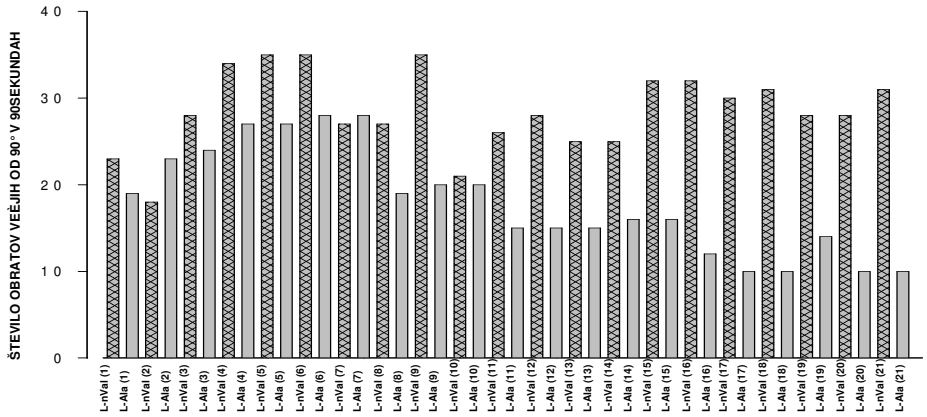


Slika 15: Vedenjski odzivi ameriških somičev z majhnimi regeneriranimi vohalnimi organi na aminokisline. Zvezdice označujejo značilno razliko med odzivi na pogojno aminokislino L-nVal in nepogojno aminokislino (Wilcoxonov test predznačenih rangov; $p<0.05$).

(Fig. 15 Responses to amino acid stimuli in bullhead catfish with small regenerated olfactory organs conditioned to L-nVal. Stars indicate significant difference between responses to conditioned and non-conditioned amino acids (Wilcoxon sum of ranks test; $p<0.05$).)



D) RAZLIKOVALNI TRENING

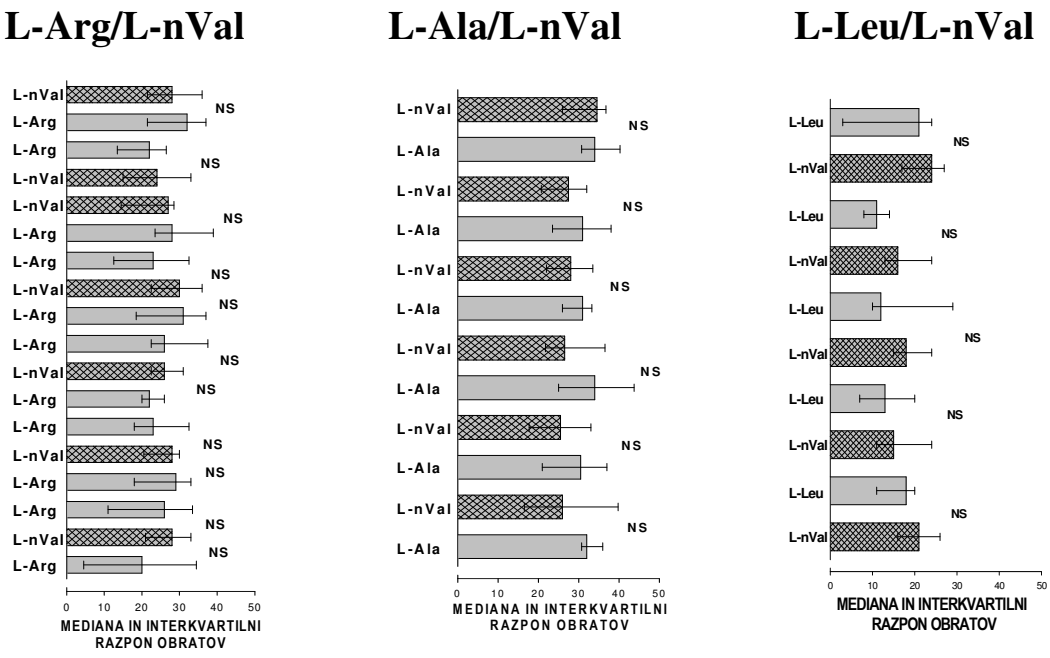


Slika 16: Somič z majhnimi regeneriranimi lamelami (**A, B**). Elektrofiziološki odzivi levega vohalnega organa (**B**) na aminokisline (**C**); prikaz in statistika na teh slikah sta enaka kot na sliki 14. (**D**) L-nVal/L-Ala razlikovalni trening.

(Fig. 16 Catfish with small regenerated lamellae (**A, B**). EOG responses of left olfactory organ to amino acids (**C**) - graphs and statistics are the same as on Fig. 14. L-nVal/L-Ala discrimination training (**D**).)

4.3.4 Vedenjski odzivi anozmičnih somičev na aminokisline

Anozmični somičev (N=8) s prstastimi lamelami ali izrastki iz epitela in veziva (Slika 10 in 11) namesto vohalnih organov, nismo mogli pogojiti na L-nVal. Sedemnajst mesecev po izrezanju vohalnih rozet in po 169-212 predstavitvah L-nVal, ti somiči niso razlikovali L-nVal od ostalih aminokislin (Slika 17). Anozmični somiči so se na ponavljajoče predstavljeno aminokislino L-nVal odzvali enako intenzivno kot na aminokisline L-Ala, L-Arg in L-Leu.



Slika 17: Nesposobnost razlikovanja aminokislin L-Arg in L-nVal, L-Ala in L-nVal ter L-Leu in L-nVal (N=8). NS = neznačilna razlika (Wilcoxonov test predznačenih rangov; $p<0.05$).

(Fig. 17 Lack of olfactory discriminations between L-Arg and L-nVal, L-Ala and L-nVal and L-Leu and L-nVal (N=8) (NS stands for non-significant; Wilcoxon sum of ranks test; $p<0.05$).)

5 RAZPRAVA IN SLKEPI

5.1 RAZPRAVA

Vohalne celice so primarne živčne celice, ki jih mlade vohalne celice nadomeščajo z skozi celotno življenje (Graziadei in Monti Graziadei, 1979; Suzuki in Takeda 1991; Schwob in sod., 1994; Caggiano in sod., 1994; Huard in sod., 1998; Ohta in Ichimura, 2001; Carter in sod., 2004; Schwob, 2005). Po poškodbah vohalnega epitela (Cancalon, 1982, 1983b; Hansen in sod., 1994; Valentinčič in sod., 2000; Iwema in sod., 2004) in po prerezanju vohalnega živca (Nordlander in Singer, 1973; Cancalon in Elam, 1980; Cancalon, 1987; Morison in Costanzo, 1989; Zelinski in Hara, 1992; Hoyk in sod., 1993) vohalni organ regenerira. Po poškodbah vohalnega epitela se različni tipi novih vohalnih celic povežejo z istimi območji vohalnega bulbusa kot v intaktnih vohalnih organih (Cummings s sod., 2000; Sengoku s sod.; 2001), medtem ko se po prerezanju vohalnega živca nove vohalne celice, ki izražajo enake vohalne receptorje, povežejo z več kot enim glomerulom (Costanzo, 2000, 2005). Pomembno vlogo pri regeneraciji vohalnega organa igrajo glia celice, ki jih ob prerezanju vohalnega živca poškodujejo (Williams in sod., 2004; Costanzo, 2005; Li in sod., 2005). Glia celice ne regenerirajo (Li in sod., 2005). Mnoge študije so pokazale, da presaditev glia celic vohalnega sistema na mesto poškodbe perifernega in centralnega živčnega sistema omogoči uspešnejšo regeneracijo živčnih celic (Li in sod., 1998; Ramon-Cueto in sod., 1998; Verdu in sod., 1999; Imazumi in sod., 2000; Bartolomei in Greer, 2000; Franklin in Barnett, 2000; Ramon-Cueto in sod., 2000; Lu in sod. 2001; Lu in sod., 2002; Ramer in sod., 2004). Proučevanje vohalnega organa in mehanizmov regeneracije pripomore k razumevanju pogojev, ki omogočajo regeneracijo živčnih celic v perifernem in centralnem živčevju (Bartolomei in Greer, 2000).

5.1.1 Obseg regeneracije vohalnega organa

Vohalne rozete ameriškega somiča (*Ameiurus melas*) sestavlja 24-34 vohalnih lamel (Stenovec, 2001). Če odstranimo celotno vohalno rozeto, odstranimo tudi plast izvornih vohalnih celic, vohalni organ ne regenerira (Valentinčič in sod., 1994, 2000). Po kauterizaciji (izžigu) vohalnih rozet vohalni organ cebric tudi ni regeneriral (Byrd, 2000; Vankirk in Byrd; 2003). Pogoj za regeneracijo vohalnega organa je prisotnost nepoškodovanih bazalnih celic. Po kemični poškodbi vohalnega epitela večina vohalnih celic propade; bazalne celice ostanejo nepoškodovane (Cancalon, 1982, 1983b; Burd, 1993; Hansen in sod., 1994; Ducray in sod., 2002; Iwema in sod., 2004). Kemično poškodovan vohalni epitel regenerira in je po mesecu dni po obsegu in zgradbi podoben intaktnemu vohalnemu epitelu. Z delnim izrezanjem vohalnih rozet smo v nosni votlini zmanjšali število bazalnih celic. Iz majhnih delov dvanajstih do osemnajstih vohalnih lamel tik ob rafi so regenerirale majhne popolne vohalne rozete ali pahljačaste vohalne rozete (Slika 4 in 5). Regenerirane rozete so vsebovale 13-22 vohalnih lamel. Če smo po delnem izrezanju vohalnih rozet pustili majhne dele treh do sedmih vohalnih lamel tik ob rafi, so regenerirale deformirane vohalne rozete z dva do dvanajstimi vohalnimi lamelami (Slika 9); pogosto so se na mestu prvotne vohalne rozete oblikovale prstaste lamele (Slika 10) ali nepravilni izrastki iz veziva in epitela (Slika 11), ki niso delovali kot vohalni organ. Obseg regeneracije vohalnega organa je hipotetično odvisen od števila zarodnih celic vohalnega epitela, ki ostanejo po izrezanju v nosni votlini.

5.1.2 Živčne povezave regeneriranih vohalnih rozet z vohalnim bulbusom

Tako kot intakten vohalni epitel so tudi regenerirane vohalne rozete vsebovale dolge vohalne celice s cilijami in vohalne celice srednjih dolžin z mikrovili. Njihovi aksoni so segali v ista področja vohalnega bulbusa kot pri intaktnih vohalnih organih. Regenerirane vohalne rozete ameriških somičev so se elektrofiziološko odzivale na aminokisline in so omogočale vohalno razlikovanje aminokislin (Stenovec, 2001).

Pri ribah vohalne celice s cilijami in mikrovili ter kriptne celice konvergirajo v različna območja vohalnega bulbusa (Morita s sod., 1996; Morita in Finger, 1998; Hansen s sod., 2003, 2005). Različni tipi vohalnih celic se odzivajo na različne skupine vonjav: aminokisline, nukleotide in žolčne kisline (Hansen s sod., 2003, 2005; Nikonov in Caprio, 2001). Pri ameriškem somiču so aksoni dolgih vohalnih celic s cilijami konvergirali v posteriorno ventralno, anteriorno in lateralno dorzalno območje vohalnega bulbusa (Slika 7 A, B), medtem ko so aksoni vohalnih celic srednjih dolžin z mikrovili konvergirali v anteriorno dorzalno, mediano, posteriorno in lateralno ventralno območje vohalnega bulbusa (Slika 7 A, C). Pri kanalskem somiču so aksoni dolgih vohalnih celic s cilijami konvergirali v ventralno in mediano območje vohalnega bulbusa, medtem ko so aksoni vohalnih celic srednjih dolžin z mikrovili konvergirali skoraj izključno v dorzalno območje vohalnega bulbusa (Hansen s sod., 2003, 2005). Dolge vohalne celice kanalskega somiča so se odzivale na amino kisline in žolčne kisline, medtem ko so se vohalne celice srednjih dolžin odzivale na aminokisline in nukleotide (Hansen s sod., 2003, 2005; Nikonov in Caprio, 2001). Kriptne celice smo pri ameriškem somiču opazili po vstavitvi DiI kristalov v lateralno ventralno območje vohalnega bulbusa v vohalnih organih dveh rib (Slika 7A, D). Sklepamo, da so pri ameriškem somiču kriptne celice povezane z majhnim, omejenim območjem lateralno ventralnega predela vohalnega bulbusa. Kriptne celice kanalskega somiča so bile povezane z majhnima območjema anteriorno ventralnega in posteriorno ventralnega vohalnega bulbusa (Hansen s sod., 2003).

Ameriškim somičem z regeneriranimi vohalnimi rozetami smo vsadili DiI kristale v anteriorno in lateralno območje ventralnega vohalnega bulbusa. Pri kanalskem somiču in cebricah se ta območja vohalnega bulbusa odzivajo izključno na aminokisline (Nikonov in Caprio, 2001; Friedrich in Korsching, 1997). Regenerirani vohalni organi ameriškega somiča so, enako kot intaktni vohalni organi, vsebovali dolge vohalne celice s cilijami, njihovi aksoni so konvergirali v anteriorno ventralno območje vohalnega bulbusa (Slika 8 A, B) in vohalne celice srednjih dolžin z mikrovili, njihovi aksoni so konvergirali v lateralno ventralno območje vohalnega bulbusa (Slika 7 A, C). Tudi po kemični poškodbi vohalnega epitela mišk so se povezave med vohalnim

epitelom in vohalnim bulbusom obnovile (Cummings in sod., 2000; Sengoku in sod.; 2001). V regeneriranih vohalnih organih mišk so se aksoni novih vohalnih celic ponovno povezali z istimi območji vohalnega bulbusa, kot pri intaktnih vohalnih organih. V vohalnih organih mišk, ki so regenerirali po prekinitvi vohalnega živca, so vohalne celice, ki so izražale P2 vohalni receptor, konvergirale v več kot en glomerul. P2 glomeruli v regeneriranih vohalnih organih miši so bili razpršeni po celotni površini vohalnega bulbusa (Costanzo, 2000, 2005). Pomembno vlogo pri regeneraciji vohalnega organa imajo ovojne celice in fibroblasti (Williams in sod., 2004; Li in sod., 2005), ki se ob prerezu vohalnega živca poškodujejo (Li in sod., 2005; Costanzo, 2005). Ovojne celice in fibroblasti ne regenerirajo (Li in sod., 2005). Znano je, da vohalni organ po prekinitvi živca pogosto ne regenerira (Costanzo, 2005).

5.1.3 Majhni regenerirani vohalni organi

5.1.3.1 Regenerirane vohalne lamele in živčne povezave z vohalnim bulbusom

Majhne deformirane vohalne rozete z dva do dvanajstimi vohalnimi lamelami so vsebovale dolge vohalne celice s cilijami in vohalne celice srednjih dolžin z mikrovili. Enako kot v regeneriranih vohalnih rozetah so v majhnih regeneriranih vohalnih organih z nekaj lamelami aksoni novih vohalnih celic segali v ista območja vohalnega bulbusa, kot pri intaktnih vohalnih organih. Tudi pri miših so po poškodbi vohalnega epitela nove vohalne celice konvergirale v ista območja vohalnega bulbusa, kot pri intaktnem vohalnem organu (Cummings in sod., 2000; Sengoku in sod.; 2001). Pokazali smo, da po poškodbah vohalnega epitela vohalni organ, ne glede na obseg regeneracije, elektrofiziološko kot tudi vedenjsko deluje normalno (Valentinčič in sod., 2000; Stenovec, 2001). Elektrofiziološko so se tako regenerirane vohalne rozete (Stenovec, 2001) kot tudi majhne vohalne lamele odzivale na širok spekter aminokislin, celo na šibko učinkovita dražljaja L-glicin in L-prolin. Enostranska regeneracija vohalnih rozet (Valentinčič in sod., 2000; Stenovec, 2001) ali le nekaj majhnih vohalnih lamel je somičem omogočala razlikovanje aminokislin.

5.1.3.2 Elektroolfaktogram (EOG) majhnih regeneriranih vohalnih organov

18 mesecev po odstranitvi vohalnega organa so bile amplitude EOG različnih aminokislin v majhnih regeneriranih vohalnih organih za več kot 60% manjše od EOG amplitud v intaktnih vohalnih organih. Pri šarenki so bile 7-8 mesecev po prekinitvi vohalnega živca EOG amplitude v regeneriranih vohalnih organih 50% manjše od tistih v intaktnih vohalnih organih (Evans in Hara, 1985). Pri žabi so opazili zmanjšanje amplitud EOG, vendar so se v enem mesecu po prekinitvi vohalnega živca EOG amplitude v regeneriranih vohalnih organih žab povečale do prvotnega stanja (Troitskaia, 1987). Pri zlati ribici (Zippel in sod., 1993) in pri golobu (Oley in sod., 1975; Bedini in sod., 1976) so bile po prekinitvi vohalnega živca amplitude EOG odzivov v regeneriranih vohalnih organih podobne amplitudam EOG odzivov v intaktnih vohalnih organih. Hipotetično so EOG odzivi seštevek receptorskih potencialov vohalnih celic (Ottoson, 1971; Caprio, 1980; Silver, 1982; Valentinčič in sod., 2005; Scott in Scott-Johnson, 2002) iz česar lahko sklepamo, da so majhne EOG amplitude v regeneriranih vohalnih organih posledica majhnega števila vohalnih celic. V majhnih regeneriranih vohalnih organih somiča z nekaj lamelami so bile, enako kot v regeneriranih vohalnih rozetah somiča (Stenovec, 2001), relativne EOG amplitude (glede na L-alanin) izzvane z aminokislinami podobne relativnim EOG amplitudam v intaktnih vohalnih organih (Slika 14B, D in 16C), kar nakazuje na ekspresijo podobnih razmerij specializiranih vohalnih celic v intaktnih in majhnih regeneriranih vohalnih organih.

5.1.3.3 Razlikovanje aminokislin pri somičih z majhnimi vohalnimi organi

Voh omogoča razlikovanje vonjav; celo semi-ozmični somiči, somiči z eno vohalno rozeto, so razlikovali aminokisline (Valentinčič, 1994, 2000). Pokazali smo, da vohalne celice v majhnih vohalnih organih z 2-12 lamelami omogočajo razlikovanje aminokislin.

Po delni odstranitvi vohalnih rozet so bili somiči sprva anozmični in niso razlikovali aminokislin (Valentinčič in sod., 2000; Stenovec, 2001). Vohalne sposobnosti so se po

regeneraciji vohalnih organov povrnile (Valentinčič in sod., 2000; Stenovec, 2001). Somiči z regeneriranimi vohalnimi rozetami so razlikovali aminokisline 6 mesecev po operaciji (Stenovec, 2001), medtem ko so somiči z enostransko regeneracijo nekaj vohalnih lamel začeli razlikovati aminokisline osem do dvajset mesecev po operaciji. Somič z najmanjšim vohalnim organom, z dvema lamelama, je začel razlikovati aminokisline 20 mesecev po operaciji (Slika 16D); ko je bila razlikovalna sposobnost vzpostavljena, je bil plavalni odziv somiča na pogojno aminokislino dvakrat večji kot na nepogojno aminokislino. Odziv somiča na nepogojno aminokislino se je zmanjšal naenkrat, kar spominja na AHA izkušnjo. Somiči z majhnimi regeneriranimi vohalnimi organi so razlikovali pogojno aminokislino L-nVal od vseh ostalih aminokislin (Slika 15), kar kaže na ponovno vspostavitev vseh funkcij vohalnega epitela. Somiči z regeneriranimi vohalnimi rozetami so razlikovali aminokisline enako ali celo bolje kot isti somiči pred izrezanjem vohalnih rozet (Stenovec, 2001). Tudi hrčkom se je sposobnost vohalnega razlikovanja po prekinitvi vohalnega živca povrnila (Yee in Costanzo, 1995, 1998). Vprašanje je ali omogočajo majhni vohalni organi somiča z nekaj lamelami razlikovanje zmesi vonjav.

5.1.3.4 Nedelujoča regenerirana tkiva, ki so zrasla na mestu prvotnih vohalnih rozet

Iz kratkih delov treh do sedmih vohalnih lamel in rafe so v manj kot 20% primerov nastale majhne deformirane vohalne rozete; v 80% primerov so namesto vohalnega organa zrasle prstaste lamele in nepravilni izrastki iz epitela in veziva. Prstaste lamele niso tvorile živčnih povezav z vohalnim bulbusom, ti organi niso delovali kot vohalni epitel. Prstaste lamele in nepravilni izrastki epitela in veziva se elektrofiziološko niso odzivali na aminokisline in somičem niso omogočala razlikovanja aminokislin. Ti somiči so bili anozmični. Za regeneracijo vohalnega organa je hipotetično potrebna zadostna količina bazalnih celic.

5.1.3.5 Vedenjski odzivi anozmičnih somičev na aminokisline

Okus ne omogoča razlikovanja aminokislin, kljub temu sproža prehranjevalno vedenje (Valentinčič in sod., 1994, 2000). Anozmični somiči brez vohalnih organov so se na aminokislini L-Ala in L-Arg odzivali z enako intenziteto plavanja. Pri somičih z nedelujočimi regeneriranimi tkivi namesto vohalnih organov tudi ni razlikovanja okušalnih dražljajev. Ti anozmični somiči so se na ponavljajoče predstavljeno aminokislino L-nVal odzvali enako intenzivno kot na L-Ala, L-Arg in L-Leu (Slika 17). Pri kanalskem somiču sta L-Ala in L-Arg visoko učinkovita okušalna dražljaja, medtem ko je L-Leu šibko učinkovit okušalni dražljaj (Caprio, 1975). Prisotnost nadpražnega okušalnega dražljaja pri anozmičnih somičih sproža iskanje hrane.

5.2 SKLEPI

Ugotovili smo, da je obseg regeneracije vohalnega organa ameriškega somiča odvisen od površine vohalnega epitela, ki ostane po operaciji v nosni votlini. Iz delov dvanajstih do osemnajstih vohalnih lamel in rafe so regenerirale majhne popolne in majhne pahljačaste vohalne rozete, medtem ko so iz majhnih delov treh do sedmih vohalnih lamel in rafe regenerirale deformirane vohalne rozete z dvema do dvanajstimi vohalnimi lamelami. V večini primerov so imela regenerirana epitelna in vezivna tkiva nepravilno obliko in niso delovala kot vohalni organ. Kadar smo odstranili celotno vohalno rozeto vohalni organ ni regeneriral, bodisi da je kirurško odprtino zarasla koža ali pa je odprtino pokrilo vezivo in tanek epitel.

Po regeneraciji vohalnih organov se živčne povezave med vohalnim epitelom in vohalnim bulbusom obnovijo. V vohalnem epitelu intaktnih in regeneriranih vohalnih organov smo z barvilom DiI retrogradno iz vohalnega bulbusa obarvali dolge vohalne celice s cilijami, njihovi aksoni so segali v anterorno ventralni del vohalnega bulbusa, in vohalne celice srednjih dolžin z mikrovili, njihovi aksoni so segali v lateralno ventralni del vohalnega bulbusa.

Regenerirani vohalni organi so bili ne glede na velikost popolnoma funkcionalni (Stenovec, 2001). Najmanjše regenerirane vohalne organe so sestavljale dve do dvanajst vohalnih lamel. Ti vohalni organi so se odzivali na širok spekter aminokislin. Relativne EOG amplitude aminokislin so bile v majhnih vohalnih organih podobne kot pri intaktnih vohalnih organih. Somiči z enostransko regeneracijo majhnih vohalnih organov so razlikovali aminokisline.

6 POVZETEK (SUMMARY)

6.1 POVZETEK

Vohalne rozete ameriškega somiča (*Ameiurus melas*) sestavlja 24-34 vohalnih lamel, ki so razporejene okoli osrednje rafe. Po delnem izrezanju vohalne rozete, ta regenerira do različnih velikosti in oblik; od majhne popolne vohalne rozete, pahljačaste rozete do nekaj posameznih vohalnih lamel. Obseg regeneracije vohalnega organa je odvisen od površine vohalnega epitela, ki po operaciji ostane v nosni votlini. Iz majhnih delov dvanajstih do osemnajstih vohalnih lamel in rafe so regenerirale bodisi majhne popolne vohalne rozete bodisi vohalne rozete pahljačastih oblik. Iz majhnih delov treh do sedmih vohalnih lamel in rafe so regenerirale deformirane vohalne rozete z nekaj majhnimi lamelami, pogosto so namesto vohalne rozete nastala samo vezivna in epitelna tkiva, ki niso imela oblike vohalne rozete. Po popolni odstranitvi vohalnega organa se votlina počasi zaraste koža ali vezivom, ki ga pokrije tanka plast epitela.

Majhne popolne in pahljačaste vohalne rozete so vsebovale 13-22 vohalnih lamel z vohalnimi celicami, ki so se nahajale ob rafi. Preverili smo ali se po regeneraciji vohalnih rozet razporeditev živčnih povezav med vohalnim epitelom in vohalnim bulbusom obnovi. Z uporabo barvila DiI smo retrogradno, iz vohalnega bulbusa, v vohalnem epitelu intaktnih in regeneriranih vohalnih rozet obarvali različne vohalne celice. V intaktnih vohalnih organih so se dolge vohalne celice s cilijami obarvale po vnosu DiI kristalov v posteriorno dorzalno, lateralno dorzalno ter anteriorno območje vohalnega bulbusa, medtem ko so se vohalne celice srednjih dolžin z mikrovili obarvale po vnosu DiI kristalov v lateralno ventralno, anteriorno dorzalno, mediano in posteriorno območje vohalnega bulbusa. Kriptne celice smo opazili le po vnosu DiI kristalov v lateralno ventralno območje vohalnega bulbusa. V regeneriranih vohalnih rozetah so bile, enako kot v intaktnih rozetah, dolge vohalne celice povezane z anteriornim ventralnim območjem vohalnega bulbusa, medtem ko so bile vohalne celice srednjih dolžin povezane z lateralnim ventralnim območjem vohalnega bulbusa.

Regenerirane vohalne rozete so se elektrofiziološko odzivale na aminokisline in so somičem omogočale razlikovanje aminokislin (Stenovec, 2001).

Iskali smo najmanjše regenerirane vohalne organe, ki omogočajo vohalno razlikovanje aminokislin. Iz majhnih delov treh do sedmih vohalnih lamel in rafe so nastale majhne deformirane vohalne rozete z nekaj vohalnimi lamelami; pogostokrat so zrasla nedelujoča tkiva. Deformirane vohalne rozete so vsebovale dve do dvanajst lamel. Ti majhni vohalni organi so imeli povezave z vohalnim bulbusom. Dolge vohalne celice s cilijami so bile povezane z anteriorno ventralnim območjem vohalnega bulbusa, medtem ko so bile vohalne celice srednjih dolžin z mikrovili povezane z lateralno ventralnim področjem vohalnega bulbusa. Mali vohalni organi so se elektrofiziološko odzivali na širok spekter aminokislin, celo na šibek vohalni dražljaj L-prolin. V intaktnih in majhnih regeneriranih vohalnih organih so bile relativne (glede na L-alanin) EOG amplitude aminokislin podobne; visoko učinkovita L-Met in L-Cys sta prožila velike EOG amplitude, medtem ko sta nizko učinkovita L-Val in L-Pro prožila majhne EOG amplitude. Testirali smo razlikovanje aminokislin pri somičih z majhnimi regeneriranimi vohalnimi organi. Pogojili smo jih na L-norvalin. Osem mesecev po odstranitvi vohalnih rozet je začel prvi somič z enostransko regeneracijo vohalnega organa razlikovati aminokisline; 12 mesecev po operaciji so štirje somiči razlikovali aminokisline. Somič z le dvema vohalnima lamelama je začel razlikovati aminokisline 20 mesecev po operaciji. Somiči z majhnimi regeneriranimi vohalnimi organi so se na pogojno aminokislino L-norvalin odzvali z vsaj dvakrat večjo intenziteto plavanja kot na ostale aminokisline. Regenerirani vohalni organi z nekaj lamelami so somičem omogočali razlikovanje aminokislin.

V manj kot 20 % primerov so iz majhnih delov treh do sedmih vohalnih lamel in rafe regenerirale deformirane vohalne rozete; v večini primerov so zrasle prstaste lamele ali nepravilni izrastki, ki sta jih sestavljala vezivo in epitel. Prstaste lamele niso bile povezane z vohalnim bulbusom. Nedelujoča regenerirana tkiva se elektrofiziološko niso odzivala na aminokisline in niso omogočala razlikovanja aminokislin. Anozmični somiči so se na ponavljajoče predstavljen okušalni dražljaj L-norvalin odzvali z

enakim ali celo manj intenzivnim iskanjem hrane kot na najbolj učinkovita okušalna dražljaja L-alanin in L-arginin.

6.2 SUMMARY

Intact olfactory rosetae of black bullhead catfish (*Ameiurus melas*) contain 24-34 olfactory lamellae, which are attached to the middle rapha. After partial excision of the olfactory rosetae complete or limited regeneration occurred. The size of the regenerated olfactory organ depended upon the size of the remaining olfactory epithelium. From small portions of twelve to eighteen remaining olfactory lamellae with rapha either small complete or fan-like olfactory rosetae regenerated. Small portions of three to seven remaining olfactory lamellae with rapha allowed regeneration of deformed olfactory rosetae with only few small olfactory lamellae; in many cases un-organized tissues emerged. After total excision of the olfactory rosetae olfactory organs did not regenerate.

The small complete and fan-like olfactory rosetae contained 13-22 olfactory lamellae with ORNs that were situated in the sensory epithelium near rapha. We visualized nerve connections between the olfactory epithelium and the olfactory bulb (OB) inserting retrograde tracer DiI into appropriate positions of the olfactory bulb. In intact olfactory organs tall ciliated ORNs were labeled after insertions of DiI crystals into posterior dorsal, lateral dorsal and anterior OB areas, whereas intermediate microvillar ORNs were labeled after insertions of DiI crystals into the lateral ventral, anterior dorsal, medial and posterior OB areas. Crypt (short) cells were observed only after lateral ventral OB insertion of DiI crystals. Like in intact, in regenerated olfactory rosetae tall ciliated ORNs were connected to anterior ventral OB areas, whereas intermediate microvillar ORNs were connected to lateral ventral OB areas. The regenerated olfactory roseate responded to amino acids electrophysiologically and enabled the amino acid discrimination (Stenovec, 2001).

We were looking for the smallest olfactory organs that enable olfactory discrimination. From portions of three to seven remaining olfactory lamellae small deformed olfactory rosetae with few lamellae regenerated; in most cases un-organized tissues emerged. The deformed olfactory rosetae contained two to twelve regenerated lamellae. These small olfactory organs had connections with the OB. The tall ciliated ORNs targeted anterior ventral OB areas, whereas intermediate microvillar ORNs targeted lateral

ventral OB areas. The small regenerated olfactory organs with only few lamellae responded to wide range of amino acids, including the poorly stimulatory L-proline, electrophysiologically. In intact and small regenerated olfactory organs relative (relative to L-alanine) EOG amplitudes of amino acids were similar; highly stimulatory L-Cys and L-Met, evoked large EOG amplitudes, whereas poorly stimulatory L-Val and L-Pro, evoked the small EOG amplitudes. We tested catfish with small regenerated olfactory organs for amino acid discrimination. We conditioned the catfish to L-norvaline. Eight months after the excision of olfactory roseate the first catfish with unilateral regeneration of small olfactory organ started to discriminate amino acids; 12 months after the surgery four catfish discriminated amino acids. The catfish with only two regenerated olfactory lamellae discriminated amino acids 20 months after the surgery. Each catfish with small regenerated olfactory organ responded to the conditioned L-norvaline with at least two times the amount of swimming than to the other amino acids. The regenerated olfactory organs with few lamellae enabled amino acid discrimination.

In less than 20% of cases deformed olfactory rosetae regenerated fom small portions of three to seven remaining olfactory lamellae with rapha; in 80% of cases finger-like lamellae or un-organized epithelial and connective tissues formed. Finger-like lamellae did not connect to the OB. The non-functional regenerated tissues did not respond to amino acids electrophysiologically and did not enable discrimination of amino acids. Anosmic catfish responded to repeatedly presented amino acid L-nVal equal or even less intense food searching behavior than to L-Ala and L-Arg, the most effective taste stimuli.

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8 ZAHVALA

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JANEZ,

PRIJATELJI,

THE PETER,

ter seveda JURE in TADEJA

najlepša hvala!

9 PRILOGA A

Protokol priprave vzorcev za vrstično mikroskopijo (Drašlar, ustno sporočilo)**:**

1. Fiksacija	1% glutaraldehid, 0.5% formaldehid, 0.1M kakodilatni pufer	2-4 ure
2. Spiranje	0.1M kakodilatni pufer	4 krat po 10 minut
3. Postfiksacija	1% osmijev tetraoksid, 0.1M kakodilatni pufer	1 ura
4. Spiranje	0.1M kakodilatni pufer	6 krat po 10 minut
5. Dehidracija	30% etanol	15 minut
	50% etanol	15 minut
	70% etanol	15 minut
	80% etanol	15 minut
	90% etanol	15 minut
	96% etanol, aceton v razmerju 1:1	15 minut
	aceton	2 krat po 15 minut

6. Sušenje v ogljikovem dioksidu pri kritični točki

10 PRILOGA B

Članek, ki je izšel v reviji Chemical Senses

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Correlations between Olfactory Discrimination, Olfactory Receptor Neuron Responses and Chemotopy of Amino Acids in Fishes

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Key words: behavior, coding, conditioning, electroolfactogram, electrophysiology, fishes, mixtures

Olfactory discrimination of amino acids in catfish and zebrafish

Catfishes (genera *Ictalurus* and *Ameiurus*) discriminate nearly every conditioned amino acid stimulus from every other amino acid stimulus (Valentinčič and Caprio, 1994; Valentinčič *et al.*, 1994, 2000a). However, bullhead catfish were always unable to discriminate L-isoleucine (L-Ile) from L-valine (L-Val) and in some cases they were also unable to discriminate L-alanine (L-Ala) from L-serine (L-Ser) and glycine (Gly). We discovered that the olfactory discrimination capabilities of catfish and zebrafish (*Danio rerio*) are very similar. Knowledge of chemotopic projections of amino acid stimuli on the surface of the olfactory bulb studied with calcium labeling technique (Friedrich and Korsching, 1997) enabled us to predict which amino acids zebrafish can and cannot discriminate. Differential bulbar activity patterns should facilitate olfactory discrimination, whereas identical bulbar patterns should not enable olfactory discrimination. Largely different bulbar activity patterns for short-chain neutral and long-chain neutral, acidic and basic amino acids make their behavioral discrimination easy. Nearly identical bulbar activity patterns that in most zebrafish occur after stimulation with L-Val and L-Ile did not enable discrimination of these two amino acids irrespective of the discrimination training applied. The bulbar activity patterns that occur after stimulation with chemically similar amino acids, such as L-Ala and L-Ser and L-arginine (L-Arg) and L-lysine (L-Lys) are not identical, their fractional differences allowed zebrafish to discriminate these amino acid pairs. In conclusion, minor differences in bulbar activity patterns enable olfactory discrimination of amino acids; the more similar these patterns are, the less the chance of their olfactory discrimination. In individual zebrafish, the bulbar activity patterns after stimulation with the same amino acid are slightly different (Friedrich and Korsching, 1997), which makes discrimination capabilities of individual zebrafish different. In bullhead catfish, the individual differences in L-Ala/ L-Ser discrimination capabilities depend on phenotypic expression rather than genetic differences between individuals since the same catfish that did not discriminate L-Ala from L-Ser before olfactory organ extirpation started to discriminate these two amino acids subsequent to regeneration (Stenovec and Valentinčič, 2001).

Olfactory discrimination of amino acid mixtures in bullhead catfish

Bullhead catfish initially detect binary and ternary mixtures of amino acids as their more stimulatory components; five to 12 successive comparisons of a mixture and its more stimulatory component alone enable the catfish to discriminate the more stimulatory amino acid from the mixture (Valentinčič *et al.*, 2000b). The more stimulatory components of binary mixtures were prepared using amino acid concentrations that were 3–30-fold higher than their equal stimulatory effectiveness concentrations determined by equal amplitude of electroolfactogram (EOG). Multimixtures composed of seven and 12 amino acids were studied at component concentrations that resulted in equal EOG amplitudes. Catfish discriminated a conditioned seven amino acid mixture from their six-, five- and four-component counterparts. The conditioned mixture of 12 amino acids was discriminated from its nine- and 10-component counterparts; however, irrespective of the missing component, they were unable to discriminate the 12-component mixture from its 11-component counterparts.

Regenerated olfactory organs facilitate olfactory discrimination of amino acids

To study olfactory discrimination in catfish with regenerated olfactory organs, the fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate salt (MS-222, dilution 1:8000) and olfactory organs were surgically excised (>95% of the olfactory organ was removed). The incompletely excised olfactory organs of bullhead catfish regenerated ~3 months after surgery. The olfactory discrimination capabilities of catfish were tested between 3 and 7 months post surgery. The regenerated olfactory roseta was always several times smaller than the intact olfactory roseta; in some cases, only few fingerlike lamellae had regrown. Olfactory discrimination capabilities of bullhead catfish with the intact and regenerated olfactory organs were identical and in some cases even better than the olfactory discrimination capabilities of the same catfish prior to extirpation. At the end of the 5–6 months period after surgery even a tiny single olfactory lamella enabled amino acid discrimination. We investigated the connectivity of the olfactory receptor neurons (ORNs) with different areas of the olfactory bulb (OB) in bullhead catfish with either intact or regenerated olfactory rosetae. In regener-

Ion concentrations in highly purified water

Ions	Sample 1 Highly purified water (HPW)	Sample2 HPW delivered into nasal cavity	Sample 3 Drain from the nasal cavity	Artificial pond water
Na^+	7 ± 2 µg/l	8 ± 2 µg/l	30 ± 2 µg/l	39 mg/l
K^+	50 ± 5 µg/l	64 ± 5 µg/l	45 ± 5 µg/l	4 mg/l
Ca^{2+}	5 ± 2 µg/l	16 ± 2 µg/l	12 ± 2 µg/l	7 mg/l
Mg^{2+}	0,5 ± 0,1 µg/l	2 ± 0,1 µg/l	2 ± 0,1 µg/l	2mg/l

Figure 1 Normal physiological function of olfactory receptor neurons was maintained in highly purified water. Ion concentration in highly purified water is ~1000× smaller that ion concentration in artificial pond water.

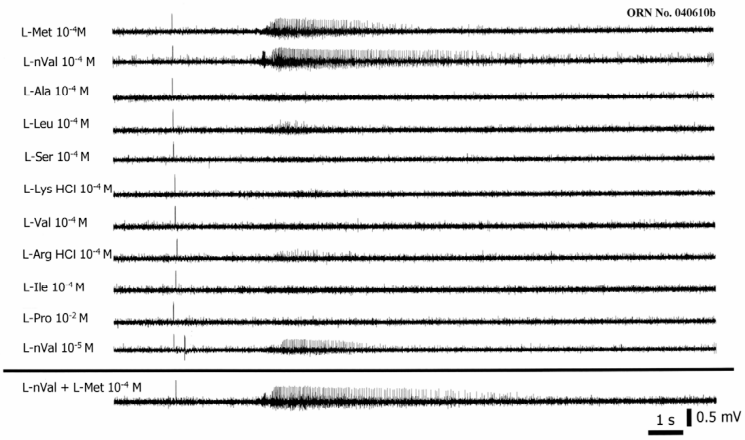

Figure 2 Greatest number of silent olfactory receptor neurons of bullhead catfish responded to two amino acids, L-methionine and L-norvaline.

ated olfactory rosetae, DiI crystals were introduced into either anterior ventral or lateral ventral sites of the bulb. In both, intact and regenerated olfactory rosetae, the insertion of the DiI into the anterior region of the ventral OB resulted in fluorescent labeling of tall (ciliated) olfactory receptor neurons (Morita and Finger, 1998; Hansen *et al.*, 2003) whereas lateral crystal insertion of the ventral OB resulted in labeling intermediate (microvillus) ORNs. Both, the fine structure of the olfactory organ and the olfactory discrimination abilities recovered during regeneration of the olfactory organs.

Electroolfactogram and single olfactory receptor neurons responses to amino acid stimuli

The cellular olfactory code is provided by a layer of ORNs. A subpopulation of ORNs is spontaneously active and a second much larger subpopulation of ORNs is not spontaneously active (silent ORNs) prior to stimulation. For technical reasons, only the physiological responses of the spontaneously active ORNs were reported previously (Kang and Caprio, 1995). The spontaneously active ORNs predominantly responded to amino acid stimuli with suppression. However neurons that code for amino acid odorants should respond to stimulation in a dose-dependent manner. For the electrophysiological experiments, we anesthetized the catfish with the anesthetic MS-222 and placed them into the recording chamber, perfused their gills with the anesthetic and immobilized them with an intramuscular injection of gallamine triethiodide (Flaxedil, 0.16 mg/100 g body wt). The concentration dependence of the suppressive response to amino acid stimuli was at best ordinal (Stevens, 1946, 1951; Velleman and Wilkinson, 1993).

We found no correlation between the ability of catfish to discriminate amino acids and the relative number of suppressive responses of ORNs. Among several hundreds of spontaneously active ORNs tested, few (<3%) cells responded to stimulation with dose-dependent excitation. Spontaneous activity is a likely property of young olfactory receptor neurons establishing synaptic connections with the olfactory bulb glomeruli.

Olfactory organs of fishes are fully functional in freshwater that contain very small ion concentrations. The physiological functions of the entire olfactory organ and of individual ORNs were preserved for several hours even in highly purified water (HPW) that contained ~1000 times fewer ions than the artificial pond water (Figure 1). Due to the high resistance (R > 18.2 MΩcm) there was little shunting of the electrophysiological signals in HPW. Responses to amino acid stimuli of numerous silent (non-spontaneously active) ORNs could be observed in these conditions. The number of lamellar locations where responses to amino acid stimuli were detected correlated highly with the amplitude of EOGs to the same stimuli. These results corroborate the assumption that the summed receptor potentials add up into the EOG amplitude. The silent ORNs responded to amino acid stimuli repeatedly and the duration of their responses was dose-dependent. Most ORNs (Figure 2) responded to one or two amino acid stimuli; the most numerous were the neurons responding to L-methionine (L-Met) and L-norvaline (L-nVal; 27% of all the tested cells). Responses to stimuli that elicit very small magnitude EOGs, such as L-proline (L-Pro), were also detected. At 23 recording locations, responses to four to eight amino acids were observed; some of these activities originated from single ORNs, whereas in other locations, single cell origin of action potentials could not be confirmed in our extracellular recordings.

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11 PRILOGA C

Rokopis za članek o regeneriranih vohalnih rozetah ameriškega somiča, ki so popolnoma funkcionalne.

REQUIRED SIZE OF REGENERATED OLFACTORY ORGAN OF CATFISH TO ENABLE OLFACTORY DISCRIMINATION

ABSTRACT

Excisions of the olfactory organs of black bullhead catfish (*Ameiurus melas*) are followed by regeneration. In this study we searched for the smallest amount of olfactory epithelium required for functional regeneration of the olfactory organ. When, after the excision, mid-portions of three to seven olfactory lamellae with the middle rapha remained *in situ,* few small olfactory lamellae developed from the remaining olfactory epithelium. After the extensive excision of 26 olfactory organs (13 catfish), only five olfactory organs in five catfish were functional. The functional olfactroy organs consisted of 2-12 short olfactory lamellae. To visualize connections between the regenerated olfactory epithelium and the olfactory bulb (OB) we labelled axons of olfactory receptor neurons (ORNs) in the OB using tracer DiI. In both, intact and regenerated olfactory organs, tall (ciliated) ORNs were connected to the anterior-ventral OB area, whereas intermediate (microvillar) ORNs were connected to the lateral-ventral OB. We also examined electrophysiological responses of the regenerated organs to amino acids. Amino acids released measurable electroolfactogram (EOG) amplitudes only in the five functional olfactory organs determined also to be functional in behavioral experiments. Highly stimulatory amino acids such as L-Met and L-Cys evoked large EOG amplitudes, whereas poorly stimulatory L-Pro evoked small EOG amplitudes. In behavioral tests the catfish with electrophysiologically functional olfactory organs responded to the conditioned amino acid L-nVal significantly more than to any other amino acid. In spite of their small size the regenerated olfactory organs enabled the catfish to discriminate amino acids. Of the 21 electro-physiologically non-functional olfactory organs some contained 0 and others up to 9 lamellae. These organs were not connected to the OB, the catfish were anosmic. The anosmic catfish responded behaviorally to amino acid stimuli detected by the taste system. The taste system alone did not enable amino acid discrimination. The anosmic catfish responded to the repeatedly presented stimulus L-nVal equally well or less than to the test stimuli such as L-alanine and L-arginine.

INTRODUCTION

Fish olfactory organs contain numerous microvillar and ciliated olfactory receptor neurons (ORNs). Crypt cells, which are co-distributed over the entire olfactory epithelium, supposedly also serve chemosensory function (Lowe and MacLeod, 1975; Caprio and Raderman-Little, 1978; Cancalon, 1978, 1983; Ichikawa and Ueda, 1977; Yamamoto, 1982; Hansen and Zeiske, 1998; Hansen and Finger, 2000). During growth the ORNs axons successively connect to the mitral cells in the OB glomeruli (Hansen and Zeiske, 1993; Dynes and Ngai, 1998; Whitlock and Westerfield, 1998; Li et al., 2005). In zebrafish most glomeruli are spherical assemblies, few glomeruli form glomerular plexi and glomerular clusters (Baier and Korsching; 1993). In catfish and goldfish microvillar ORNs, ciliated ORNs and crypt cells project to different OB areas (Morita et al., 1996; Morita and Finger, 1998; Hansen et. al., 2003; Hansen et al., 2005). ORNs expressing the same type(s) of olfactory receptor(s) (ORs) converge to topically arranged clusters of glomeruli (Sato et al., 2007). In adulthood progenitor cells retain their capacity to form new ORNs (Graziadei and Monti Graziadei, 1978, 1979, Caggiano et al., 1994; Huard et al., 1998; Carter et al., 2004; Schwob, 2005). The ORNs turnover has a consequence of repeated re-connections between ORNs and OB neurons (Graziadei and Monti Graziadei, 1978, 1979). ORNs axons converge to specific glomeruli thus maintaining the topical OB map. In OB different odorants evoke different, in many cases overlapping, glomerular activity patterns (Costanzo and

Mozell, 1976; Kauer, 1988; Friedrich an Korsching, 1997, 1998; Rubin and Katz, 1999; Uchida et all, 2000; Nikonov and Caprio, 2001; Wachowiak and Cohen; 2001; Wachowiak et al., 2002). The differential glomerular activity patterns play an essential role in odorant discrimination (Kauer and White, 2001; Schild and Riedel, 1992; Buck, 1996; Kajiya et al., 2001; Valentincic et al., 2005). Zebrafish and catfish discriminate amino acids that elicit differential glomerular patterns and can not discriminate amino acids such as L-Val and L-Ile that elicit similar or equal glomerular patterns (Valentincic et al., 2000; Valentincic et al., 2005).

Lesions of either the olfactory epithelium or the olfactory nerve provoke ORNs degeneration, which are followed by a growth of new ORNs and their re-connection to the OB neurons (Cancalon, 1982, 1983; Hansen et al., 1994; Iwema et al., 2004; Nordlander and Singer, 1973; Cancalon and Elam, 1980; Cancalon, 1987; Morison and Costanzo, 1989; Zelinski and Hara, 1992; Hoyk et al., 1993). If the entire progenitor cell layer is destroyed the olfactory epithelium does not regenerate (Byrd, 2000; Valentincic et al., 1994a, 2000; Vankirk and Byrd; 2003; Costanzo, 2005). Partial chemical injuries of catfish, zebrafish and rat olfactory organs resulted in their complete morphological regenerations (Cancalon, 1982, 1983; Hansen et al., 1994; Iwema et al., 2004). In rats, 3 months after the chemical injury to the olfactory epithelium, the distribution of eight different ORs across the ORNs was reestablished (Iwema et al., 2004). Electrophysiological studies also showed that after regeneration the olfactory organ reestablished their original function (Evans and Hara, 1985; Simmons and Getchell, 1981; Costanzo, 1985; Koster and Costanzo, 1996; Zippel et al., 1993; Oley et al., 1975; Troitskaia, 1986). In the rainbow trout olfactory nerve transection resulted in temporary loss of the electrophysiological response and its recovery after 84 days (Evans and Hara, 1985). In mice, during recovery from olfactory nerve transection the P2 receptor ORNs axons unexpectedly converged to more than one glomerulus (Costanzo, 2000, 2005). After chemical injury to the mice olfactory epithelium the young ORNs axons re-connect to the same areas of the OB as intact ORNs (Cummings et al., 2000; Sengoku et al.; 2001). Olfactory ensheathing cells and olfactory nerve fibroblasts seem to have an important role in olfactory organ regeneration (Williams et al., 2004; Li et al., 2005). During the regeneration the ensheathing cells and fibroblasts maintain open channels between olfactory epithelium and OB. Olfactory ensheathing cells and olfactory nerve fibroblasts do not regenerate.

Intact bullhead catfish discriminate almost all amino acids, whereas anosmic catfish are unable to discriminate the same amino acids (Valentincic et al., 1994a, 2000). Partial excision of the black bullhead catfish olfactory rosetae resulted in a loss of olfactory discrimination capabilities, these capabilities were regained after the regeneration of the olfactory organs (Valentincic et al., 2000). Even very small regenerated fan-like olfactory organs enabled catfish to discriminate L-Ala from L-Arg. In the present study we were searching for the minimal size of the regenerated olfactory organs that allow olfactory discrimination of amino acids. We demonstrated that small olfactory organs with only 2-12 olfactory lamellae respond to wide range of amino acids electrophysiologically and enable complete olfactory discrimination of amino acids. Using tracer DiI we visualized nerve connections between the olfactory epithelium and the OB. In the regenerated olfactory lamellae ciliated and microvillous ORNs targeted the same OB areas as in intact organs.

MATERIALS AND METHODS

Animals

Black bullhead catfish (*Ameiurus melas*) were caught in Pernica ponds near Maribor, Slovenia. They were transferred into individual aerated 80 liter (50x40x40cm^3) aquaria used also for behavioral experiments. Initially the catfish were treated against skin parasites and bacteria using malachite green and 4 parts per thousand sodium chloride solution. We maintained the catfish under 12/12 hours light/dark rhythm and fed them with cod muscles daily. The catfish started to feed regularly and were completely adapted to the new environment after approximately two months.

Excision of olfactory epithelium

To excise the olfactory epithelium we anesthetized the catfish with 1:8000 water solution of MS 222 (ethyl-3-aminobenzoat methane-sulfonate, Fluka). Immobile catfish was pinned into the wax in a perfusion chamber - during the surgery the catfish gills were continuously perfused with dechlorinated tap water containing the anesthetic. The catfish body was wrapped with wet cotton towels. Under the stereomicroscope (SMZ-1; Nikon) we dissected the bone above of the nasal cavity and exposed the olfactory epithelium. The olfactory organ was excised either completely or partially. The partially excised olfactory organs contained median portions of three to seven olfactory lamellae with parts of rapha. After the surgery

the catfish were placed into heavily aerated water until they started to breathe independently; later they were replaced into the experimental aquaria.

Observations of regenerated olfactory organs

Eighteen months after the olfactory organs excision the catfish were anesthetized with MS 222 (1:8000) and transferred into a plastic chamber containing dechlorinated tap water with the anesthetic. We pinned them into the floor of the chamber and photographed the frontal part of the head. The photographs of regenerated olfactory organs were taken under the stereomicroscope (Leica) with Nikon digital camera Coolpix 4500 2 and 18 months after the surgery.

DiI labeling

Anesthetized catfish (MS 222; 1:5000) were pinned into a dissecting chamber. We perfused them trans-cordially with Fish Ringer and subsequently with 4% formaldehyde in 0.1M phosphate buffer. Formaldehyde was prepared fresh from paraformaldehyde (Merck) few hours before the perfusion. During the perfusion we flushed the nasal cavity with a buffered mixture of 0.2% glutaraldehyde and 4% formaldehyde for fast fixation of the epithelial surface. After the fixation of the epithelium we isolated the front dorsal part of the catfish head and exposed by removing the bones and cartilage the lateral or the anterior area of the ventral olfactory bulb (OB). The isolated frontal part of the head containing OB and olfactory epithelium were left in buffered 4% formaldehyde through the night. Under a visual control we inserted small DiI crystals (1, 1`-dioctadecyl-3, 3, 3`, 3`-tetramethylindo-carbocyanine, Molecular probes, Eugene, Oregon, USA) into the exposed (anterior-ventral or lateral-ventral) OB area using an entomological needle. To prevent the spread of the DiI crystals we embedded the OB into 2% agar. During three weeks of the incubation period tissue blocks were kept in buffered 4% formaldehyde in darkness at 20°C. At the end of incubation we isolated the connected olfactory epithelium and OB and embedded them in 15% gelatin. The blocks were fixed in buffered 4% formaldehyde overnight. The preparations were sliced into 50 µm sections using Vibratome (The Vibratome Company, St. Luis) and viewed under epifluorescence (Axioscope, Opton, West Germany). The olfactory epithelia were photographed with Nikon digital camera Coolpix 4500 and contrast enhanced using Photoshop.

Scanning electron microscopy (SEM)

Catfish were anesthetized with MS222 (1:5000 water dilution; Sigma) and perfused trans-cordially with Fish Ringer for a blood removal. We isolated the olfactory organs and rinsed them with 0.1M cacodilate buffer. The isolated organs were fixed in mixture of 1% glutaraldehyde and 0.5 % formaldehyde in 0.1M cacodilate buffer. The olfactory organs were than contrasted with osmium tetraoxide (OsO_4), dehydrated in a graded series of alcohol and acetone, and dried at critical point in CO_2. Dried olfactory lamellae were isolated and covered with gold. The specimens were examined with a scanning electron microscope (Jeol 840a).

Electroolfactogram (EOG) recording

We measured the EOG in the catfish 18 months after the excision of olfactory organs. We positioned the anesthetized catfish (MS 222, 1:8000) into the experimental chamber by fixing its head into plastic clamps. During the recording catfish gills were continuously perfused with dechlorinated tap water containing the anesthetic. For the stimulation of regenerated tissues we used gravity system consisted of plastic tubes (~0.8mm inner diameter) and 15 liter dechlorinated tap water reservoir (on ~1 m of a height). From the reservoir the water flow entered the tube that divided into the irrigation and stimulation tube. Water that flowed through the irrigation tube bathed the regenerated tissues between the stimulations. During the stimulation the irrigation tube enabled an equation of the pressure in a system of tubes. The irrigation and stimulation tubes reconnected with T-bore stopcock to the outlet tube. We directed the free end of the outlet tube onto the regenerated tissue using the micromanipulator (Narishige MM-33N). In water ~1mm above the regenerated tissues we positioned the recording $Ag/AgCl_2$ electrode with Ringer-agar bridge. The 10^{-4} M (10^{-2} M) dechlorinated tap water dilutions of amino acids (L-proline) were prepared fresh less than 2 hours before the

recording. With a syringe we introduced 0.5 ml of an amino acid solution through T-tube to the stimulation tube and switched the T-bore stopcock. The water flow was redirected from the irrigation to the stimulation tube. The stimulus reached the regenerated tissues in 1-3 seconds (Koce, 1999). Intervals between consecutive stimulations were 1.5 min. The EOG signal recorded with the electrode was amplified by direct-coupled amplifier (100×, Grass P18D) and displayed on a pen recorder (OmniScribe recorder; Industrial Scientific, TX, USA). With a ruler we measured the maximum height of EOG amplitude for each amino acid.

EOG data analysis

We standardized EOG responses between different amino acids with the response after stimulation with 10^{-4} M L-alanine. We compared relative EOG amplitudes triggered by different amino acids in intact and regenerated olfactory organs using Pearson's correlation coefficients (R).

Behavioral tests

Olfaction and taste

Six months after the excision of the bullhead catfish olfactory organs that left small median portions of three to seven olfactory lamellae with parts of rapha *in situ* we started behavioral experiments. We presented amino acid L-norvaline (L-nVal) to the catfish 1-5× daily. During the period of 6-20 months we tested catfish for discrimination of amino acids. Less than 30 min before the experiment we prepared $3*10^{-2}$ M water dilutions of amino acids. We delivered 2 milliliters of the stimulus solution into aquarium using Pasteur pipette directed into aerated portion of the aquarium. The air-bubbles driven water currents that rapidly

distributed the high concentration stimulus eddies across the entire aquarium. The stimulus eddies reached the catfish in <30 seconds (Valentincic and Caprio, 1994). In a control experiment we delivered dechlorinated tap water into the aquarium. During 90 seconds experiment the catfish was video-recorded (MV1, Canon) and its activity evaluated by counting number of turns greater than 90º. Catfish got a food 90 seconds after the injection of the amino acid L-nVal into the aquarium. The number of turns during control and test procedures were compared statistically using Wilcoxon sum of ranks test [$p<0.05$]; sequential experiments conducted on the same day were compared statistically.

Taste (anosmic catfish)

To prevent cross-adaptation of taste stimuli we tested only two different amino acids per day, first presented amino acid was always the less stimulatory taste stimulus of the two (Caprio, 1978).

Olfaction (osmic catfish)

Tests for olfactory discrimination of amino acids conducted on osmic catfish contained 3-5 trials a day. To the catfish we alternately presented conditioned amino acid L-nVal and the other tested amino acids.

Amino acids used in behavioral tests and EOG recordings

We used amino acids of highest purity, L-norvaline (L-nVal), L-arginine hydrochloride (L-Arg), L-methionine (L-Met), L-cysteine hydrochloride (L-Cys), L-lysine hydrochloride (L-Lys), L-norleucine (L-nLeu), L-valine (L-Val) were Sigma (Sigma Chemical, St.Luis, MO) products, whereas L-proline (L-Pro), L-leucine (L-Leu), L-alanine (L-Ala) were Fluka (Fluka Chemica-BioChemica, Switzerland) products.

RESULTS

Small regenerated olfactory organs

After the total excision of olfactory rosetae the olfactory organs did not regenerate (N=36 organs). In young catfish (<2 years old) skin overgrown the excised area (N=20), whereas in old fish (>3 years old) the excised area was covered with a connective tissue and thin epithelium (N=16). The olfactory organs regenerated only if small portion of the olfactory epithelium remained *in situ* after the excision. In two months the remaining medial portions of three to seven olfactory lamellae with parts of rapha regenerated into small incomplete rosetae (N=13); in many cases deformed epithelial and connective tissues formed (N=13). Of 26 regenerated tissues only five tissues responded to amino acids electrophysiologically (EOG) thus confirming the term regenerated olfactory organ. These functional olfactory organs consisted of 2, 4, 5, 7 or 12 olfactory lamellae. The smallest of regenerated olfactory organs consisted of two leaf- like olfactory lamellae. Most olfactory organs consisted of leaf-like and some fingerlike olfactory lamellae. The other non-functional regenerated tissues (N=21) consisted of either 1-9 fingers-like lamellae (N=8) or emergences of connective and epithelial tissue (N=13) situated in the site of previous roseta attachment.

Electroolfactogram (EOG) of small regenerated olfactory organs

18 months after the excision of olfactory organs (N=26) only five regenerated olfactory organs in five different catfish responded to amino acid stimulation with measurable EOG recordings. In these olfactory organs the EOG amplitudes to amino acid stimulation were considerably smaller [~10mV] than in intact

olfactory organs [~30mV]. However, in intact and in the regenerated olfactory organs highly stimulatory amino acids, such as L-Cys and L-Met, evoked large EOG amplitudes, whereas poorly stimulatory L-Val and L-Pro evoked small EOG amplitudes. To compare relative EOG amplitudes (relative to L-Ala amplitude) in intact and regenerated olfactory organs, we calculated Pearson's correlation coefficients (R) between EOG amplitudes for different amino acids in intact and in regenerated olfactory organs. The correlation between the relative EOG amplitudes of amino acids in intact and regenerated olfactory organs was high and significant (R= 0.79-0.94; $p<0.05$). The minimal sized regenerated olfactory organ with two lamellae had the smallest Pearson correlation coefficient, whereas the highest Pearson's coefficient was calculated for the largest of the regenerated olfactory organs that consisted of 12 olfactory lamellae.

Connections between regenerated olfactory epithelium and olfactory bulb (OB)

Using retrograde tracer DiI we visualized nerve connections between regenerated olfactory epithelium and OB. Like in intact organs, in the regenerated olfactory organs that contained either 4 or 12 olfactory lamellae, an insertion of DiI crystals into the anterior-ventral OB area labeled tall (ciliated) olfactory receptor neurons (ORNs). Somas of tall ciliated ORNs extend from the surface of the sensory epithelium to the basal lamina. In the minimal olfactory organ with two leaf-like lamellae we inserted DiI crystals into lateral-ventral OB area, intermediated (microvillar) ORNs were labeled in most cases. Somas of intermediated (microvillar) ORNs occupy second third of sensory epithelium depth and never reach the basal lamina. In non-functional regenerated tissues containing finger-like lamellae no connections between the regenerated epithelium and the OB (N=4) were observed. These lamellae were covered with kinociliated non-sensory epithelium (N=2).

Olfactory discrimination of amino acids

We started to condition bullhead catfish (N=13) with regenerated olfactory organs to L-nVal 6 months after the extensive excision of olfactory organs and continued the conditioning trials until 20 months after the surgery. After ~ 30 conditioning trials the catfish were tested for olfactory discrimination of amino acids. The first catfish stared to discriminate the conditioned from non-conditioned amino acids 8 months after the surgery. The catfish response to conditioned stimulus L-nVal was at least twice as large as the response to other amino acids. Twelve months after the surgery four of thirteen catfish discriminated L-nVal from other amino acids. All the catfish that discriminated amino acids had only one (unilateral) olfactory organ. The catfish with the smallest functional olfactory organ containing two olfactory lamellae started to discriminate amino acids twenty months after the surgery; the response to the conditioned stimulus L-nVal was immediately after the onset of discrimination twice as large as the response to the non-conditioned stimulus L-Ala.

Behavioral responses of anosmic catfish to amino acids

Anosmic catfish (N=8) with non-functional [EOG] regenerated tissues instead of olfactory organs could not be conditioned to L-nVal. 17 months after the extensive excision of the olfactory organs and after 169-212 presentations of L-nVal, these catfish did not discriminate L-nVal from the other amino acids. Anosmic catfish responded to repeatedly presented stimuli L-nVal equally well or less than to other taste stimuli L-Ala, L-Arg and L-Leu.

DISCUSSION

Small regenerated olfactory lamellae and their connections to the olfactory bulb

With an excision of the catfish olfactory rosetae, ORNs progenitor cell layer was totally removed and the olfactory organs did not regrow (this study; Valentinčič, 1994a, 2000). Total cauterization of the zebrafish olfactory rosetae also prevented their regeneration (Byrd, 2000; Vankirk and Byrd; 2003). At least a small portion of the olfactory roseta must remain *in situ* for olfactory regeneration to occur. After comprehensive excision of olfactory rosetae, which left small medial portions of olfactory lamellae 3-7 *in situ*, small deformed olfactory rosetae regenerated, in many cases non-functional tissues emerged. The deformed olfactory rosetae consisted of 2-12 small olfactory lamellae that contained tall ciliated and intermediate microvillar ORNs; their axons targeted the same OB areas as in intact olfactory organs. Tall ciliated ORNs converged into the anterior ventral OB area, whereas intermediate microvillar ORNs targeted lateral ventral OB area. The small regenerated olfactory organs were fully functional. Electrophysiologically they responded to wide range of amino acids, even to poorly stimulatory olfactory stimulus L-proline.

Behaviorally, catfish with unilaterally regenerated few small olfactory lamellae were able to discriminate amino acids.

Electroolfactogram (EOG) of the small regenerated olfactory organs

18 months after the olfactory organ excision EOG amplitudes of amino acid were less than 40% of the EOG amplitudes of the intact olfactory organs (10 versus 30 millivolts). In rainbow trout 7-8 months after the recovery from olfactory nerve transection EOG amplitudes in regenerated olfactory organs were 50% smaller than those in the intact olfactory organs (Evans and Hara, 1985). In frog the decrease in EOG amplitude was observed; however, one month after the olfactory nerve transection the EOG amplitudes in regenerated olfactory organs of frog recovered completely (Troitskaia, 1996). Since the EOG responses represent primarily, if not exclusively, a summated ORNs receptor potentials (Ottoson, 1971; Caprio, 1978, 1980; Silver, 1982; Koce and Valentinčič, 2000; Scott and Scott-Johnson, 2002) the small EOG amplitudes in regenerated olfactory organs are hypothetically a consequence of reduced numbers of ORNs. In the regenerated olfactory organs of catfish relative EOG amplitudes evoked by amino acids were similar to the relative EOG amplitudes in intact olfactory organs, which indicate expressions of similar proportions of specialized ORNs in intact and small regenerated olfactory organs.

Olfactory discrimination of amino acids in catfish with small regenerated olfactory organs

Olfaction enables discrimination of odorants; even semi-osmic catfish, with only one of the paired olfactory organs functional, discriminated amino acids perfectly (Valentinčič, 1994b, 2000). We showed that "few" ORNs in the small regenerated olfactory lamellae enable sufficient input to enable olfactory discrimination of amino acids. Eight months after the excision of olfactory roseate first catfish with unilateral regeneration of small olfactory organ started to discriminate amino acids; 12 months after the surgery four catfish discriminated amino acids. The catfish with only two regenerated olfactory lamellae discriminated amino acids 20 months after the surgery. The conditioned discrimination started within several successive comparisons; the onset of discrimination resembles an AHA experience rather than gradual increase in difference in response between the conditioned and non-conditioned amino acids. The catfish with small regenerated olfactory organs discriminated conditioned stimulus L-nVal from all other amino acids indicating a complete recovery of amino acid discrimination capabilities. The question is weather these small olfactory organs also enable perception of complex mixtures of odorants.

Non-functional regenerated tissues

Small medial portions of olfactory lamellae 3-7 allowed growth of deformed olfactory rosetae; in many cases non-functional finger like lamellae or epithelial and connective tissues formed. The finger-like lamellae did not have nerve connections to the OB; such organs were not functional. The non-functional regenerated tissues did not respond to amino acids electrophysiologically and did not enable discrimination of amino acids.

Behavioral responses of anosmic catfish to amino acids

Taste alone does not enable the discrimination of amino acids; nevertheless, it releases feeding behavior (Valentincic, 2004). In black bullhead catfish with regenerated finger-like lamellae or epithelial and connective tissues the discrimination of taste stimuli did not develop. These anosmic catfish responded to repeatedly presented amino acid L-nVal equally or even less than to L-Ala and L-Arg and equally or more than to L-Leu. In channel catfish L-Ala and L-Arg are highly stimulatory taste stimuli, whereas L-Leu is poorly stimulatory taste stimulus (Caprio, 1975). However, the presence of a suprathreshold taste stimuli released the food searching behavior.

12 PRILOGA D

Rokopis za članek o najmanjših vohalnih organih, ki omogočajo razlikovanje aminokislin.

REGENERATED OLFACTORY ROSETAE OF BLACK BULLHEAD CATFISH ARE FULLY FUNCTIONAL

ABSTRACT

Intact olfactory organs of black bullhead catfish (*Ameiurus melas*) contain olfactory rosetae composed of intermediate rapha and 24 to 34 lamellae. We excised partially the olfactory rosetae and left the mid-portions of twelve to eighteen olfactory lamella and part of rapha *in situ*. The incompletely excised olfactory organs regenerated and formed either small olfactory rosetae containing 14-22 olfactory lamellae or a fan-like olfactory organs with 13-17 lamellae. To visualize connections between the olfactory epithelium and the olfactory bulb (OB) we retrogradely, from the OB, labeled olfactory receptor neurons (ORNs) of intact and regenerated olfactory organs using the tracer DiI. In both, intact and regenerated olfactory roseate tall-ciliated ORNs labeled after the anterior-ventral DiI insertion, whereas intermediate-microvillar ORNs labeled after the lateral-ventral DiI crystal insertion. The regenerated olfactory organs were fully functional. Electrophysiologically they responded to wide range of amino acids including the poorly stimulatory Gly. The relative EOG amplitudes of amino acids correlated highly with the EOG amplitudes of the intact olfactory organs. To demonstrate behavioral function of the regenerated olfactory organs we conditioned catfish with intact and regenerated olfactory organs with either L-Ala or L-Val. The intact catfish conditioned to L-Ala discriminated it from all amino acids, except from L-Ser and Gly. After the olfactory organ regeneration the same catfish discriminated L-Ala from other amino acids including L-Ser and Gly. Intact catfish conditioned to L-Val discriminated it from all other amino acids, except from L-Ile. The same catfish with regenerated olfactory organs also discriminated L-Val from all other amino acids, and did not discriminate L-Ile from L-Val. Catfish with regenerated olfactory organs also discriminated L-Val from the binary mixtures of amino acids containing L-Val as the less stimulatory component, whereas they did not discriminate initially L-Val from its binary mixture containing L-Val as the more stimulatory component.

INTRODUCTION

Vertebrate olfactory organs contain olfactory receptor neurons (ORNs) that project their axons to the glomeruli of the olfactory bulb (OB) (Shepherd, 1991; Laberge and Hara, 2001). Glomeruli are assemblies of ORN axons that synapse with dendrites of mitral cells. Most glomeruli in zebrafish are spherical; however, some fish glomeruli form plexi and clusters (Baier and Korsching; 1993).

Olfactory receptors (ORs) are situated either on microvilli or cilia of ORNs (Yamamoto, 1982; Laberge and Hara, 2001). In vertebrates lacking vomeronasal organ, such as fish, microvillar and ciliated ORNs are co-distributed over the entire olfactory epithelium (Lowe and MacLeod, 1975; Caprio and Raderman-Little, 1978; Cancalon, 1978, 1983; Ichikawa and Ueda; 1977; Yamamoto, 1982; Hansen and Zeiske, 1998).Crypt cells are also found in the olfactory epithelium of fish (Hansen and Zeiske, 1998; Hansen and Finger, 2000; Hansen et. al., 2003). In the channel catfish and goldfish axons of microvillar ORNs, ciliated ORNs and crypt cells converge to different areas of the olfactory bulb (Morita et al., 1996; Morita and Finger, 1998; Hansen et. al., 2003; Hansen et al.; 2005). Recent study demonstrated that ORNs axons expressing one or few types of ORs converge into specifically arranged clusters of glomeruli of zebrafish (Sato et al., 2007). In rodents ORNs expressing the same type of OR converge into one or few glomeruli located close together (Vassar et al., 1994; Ressler et al., 1994; Mombaerts et al., 1996).

Different odorants evoke differential patterns of glomerular activity (Costanzo and Mozell, 1976; Kauer, 1988; Friedrich an Korsching, 1997; Friedrich an Korsching, 1998; Fuss and Korsching, 2001; Rubin and Katz, 1999; Uchida et all, 2000; Nikonov and Caprio, 2001; Wachowiak and Cohen; 2001; Wachowiak et

al., 2002). In zebrafish behavioral studies revealed that glomerular odor maps play an essential role in odorant discrimination (Valentincic et al., 2005). Zebrafish discriminated amino acids that evoked differential glomerular activity patterns, whereas they could not discriminate amino acids, such as L-valine and L-isoleucine or L-phenylalanine and L-tyrosine, which elicite similar or equal patterns of glomerular activity (Friedrich???, 2001). Bullhead catfish are also unable to discriminate L-valine from L-isoleucine (Valentincic et al., 2000a). Even more, they did not discriminate amino acid pair L-alanine and L-serine that evoked similar glomerular activity patterns in the zebrafish OB.

Throughout life span ORNs are continuously replaced with new ORNs (Graziadei and Monti Graziadei, 1978, 1979) originating from progenitor cells located at the base of sensory epithelium (Graziadei and Monti Graziadei, 1979; Schwob et al., 1994; Caggiano et al., 1994; Huard et al., 1998; Carter et al., 2004; Schwob, 2005). Final result of ORNs turnover is a continuous reconnection of synapses between ORNs and mitral cells (Graziadei and Monti Graziadei, 1978, 1979). New ORNs are also formed after mechanical, chemical or parasitic injuries (Cancalon, 1982, 1983; Burd, 1993; Hansen et al., 1994; Valentinčič et al., 2000; Ducray et al., 2002; Iwema et al., 2004) and after olfactory nerve transection (Nordlander and Singer, 1973; Oley et al., 1975; Cancalon and Elam, 1980; Cancalon, 1987; Morison and Costanzo, 1989; Zelinski and Hara, 1992; Hoyk et al., 1993). In catfish chemically destroyed olfactory epithelium regenerated after 45-55 days (Cancalon, 1982, 1983). Electrophysiological studies indicateded that the regenerated olfactory epithelium regained its function (Evans and Hara, 1985; Simmons and Getchell, 1981; Zippel et al., 1993; Oley et al., 1975; Troitskaia, 1986). In rainbow trout (*Oncorhynchus mykiss*) a nerve transection resulted in temporary loss and, after 84 days, a recovery of the EOG response (Evans and Hara, 1985). In hamster (*Mesocricetus auratus*) it was shown that after the olfactory nerve transection the axons of young ORNs re-grow into OB and re-establish synapses with the mitral cells (Costanzo, 1985; Koster and Costanzo, 1996). In the regenerated olfactory bulbs of hamster evoked potential amplitudes were smaller than in the intact olfactory organs (Koster and Costanzo, 1996). After the recovery the mouse P2 receptor ORNs axons, which terminated pre-operatively in a single locus, terminated in multiple loci distributed across a wide surface of the OB (Costanzo, 2000, 2005). During regeneration new glomeruli were formed, positions and numbers of loci were changing until 5th months after the injury. Behavioral tests in hamster demonstrated that after regeneration of a transected nerve the odorant discrimination also regenerated (Yee and Costanzo, 1995, 1998). Novel discrimination training was required to reestablish the previous levels of the olfactory discrimination.

In the black bullhead catfish a dissection of large parts of the olfactory epithelium resulted in small regenerated olfactory organs (Valentincic et al., 2000a). These organs enabled olfactory discrimination of L-alanine and L-arginine (Valentincic et al., 2000a). In the present work we investigated the function of the small regenerated olfactory organs. The small regenerated olfactory rosetae of black bullhead catfish (*Ameiurus melas*) responded to amino acids electrophysiologically and enabled amino acids discrimination. Using retrograde tracer DiI we observed nerve connections between the olfactory rosetae and OB of the regenerated olfactory organ. Ciliated and microvillar ORNs targeted the same OB areas in regenerated and intact olfactory organs.

MATERIALS AND METHODS

Animals

Bullhead catfish (*Ameiurus melas*) were obtained from a fish farm near Ptuj, Slovenia. The catfish were maintained in aerated 500 liter rotation tanks under 12/12 hours light and dark cycle. All the experimental catfish were treated for ich with malachite green. Two months prior to the behavioral experiments they were transferred into the aerated 80 liter (20x20x20cm^3) experimental aquaria. During this period the catfish learned the concept of the experimental aquarium and started to respond immediately to feeding stimuli.

Excision of olfactory epithelium

To surgically excise the olfactory epithelium the catfish were anesthetized with MS 222 (ethyl-3-aminobenzoat methane-sulfonate, purchased from Fluka) at a dilution of 1:8000, placed into the gill irrigating chamber and pinned to wax surface. The fish was wrapped into wet cotton towels. We surgically removed the roof of the olfactory cavity under the dissection microscope (SMZ-1; Nikon). Large proportion of the olfactory epithelium was extirpated leaving only small portions of twelve to eighteen olfactory lamellae (out of ~ 30 lamellae) with rapha. The catfish were resuscitated in heavily aerated water until they

started to breathe spontaneously. They were transferred into experimental aquaria and fed regularly during the olfactory organ regeneration.

Observations of the olfactory organs
Photographs of intact and regenerated olfactory organs were taken under the stereomicroscope (MZ FLIII; Leica) with Nikon digital camera Coolpix 4500. The regenerated olfactory organs were photographed 2, 4 and 7-9 months after the surgery.

DiI labeling
The anesthetized (MS 222) catfish were pinned into the dissecting chamber. The immobilized catfish were perfused trans-cordially with fish Ringer and subsequently, to preserve the olfactory epithelium, with 4% formaldehyde in 0.1M phosphate buffer. The formaldehyde solution was prepared fresh from paraformaldehyde (Merck) few hours before the experiment. During pre-fixation of the epithelial tissues we flushed the nasal cavity with a buffered mixture of 0.2% glutaraldehyde and 4% formaldehyde. We dissected the frontal dorsal part of the head and exposed one of the OB areas: the anterior-ventral, lateral-ventral, median-ventral, posterior-ventral, anterior-dorsal, lateral-dorsal, median-dorsal or posterior-dorsal OB area. The tissue blocks containing dissected parts of the heads were immersed into buffered 4% formaldehyde overnight. We inserted the small DiI crystals (1, 1`-dioctadecyl-3, 3, 3`, 3`-tetramethylindo-carbocyanine, Molecular probes) into the exposed area of the OB with the entomological needle. To prevent spreading of DiI crystals into other areas we covered the entire OB with 2% agar for the incubation period of 19-25 days. The tissue blocks were preserved in buffered 4% formaldehyde in a dark room at 20°C. After the end of the incubation we excised the olfactory epithelium with the attached OB and embedded the preparation into 15% gelatin. The gelatin blocks were fixed in buffered 4% formaldehyde overnight and cut into 50µm sections with Vibratome 1000 Plus (The Vibratome Company, St. Luis). The sections were viewed under epifluorescence microscope (Axioscope, Opton, West Germany). Photographs of intact and regenerated olfactory epithelia were taken with Nikon digital camera (Coolpix 4500) and contrasts enhanced using Photoshop.

Electroolfactogram (EOG) in regenerated olfactory organs
We transferred the anesthetized (MS 222) catfish into the experimental chamber and fastened its head into plastic clamps. During the recordings the gills of the catfish were continuously perfused with the dechlorinated tap water containing the anesthetic. Similarly to underwater EOG recordings (Silver et al., 1976) by gravity driven dechlorinated tap water flow [~18ml/min] entered through Y-tube into the irrigation or stimulation tube. The tubes were reconnected with T-bore stopcock to the outlet tube (0.8mm inner diameter). With the micromanipulator (Narishige MM-33N) we directed the tip of the outlet tube onto the regenerated olfactory organ. With a syringe we introduced 0.5 ml of an amino acid solution at 10^{-4} M concentration through T-tube to the stimulation tube. We switched the T-bore stopcock and the water flow was redirected from the irrigation to the stimulation tube. The stimulus reached the olfactory lamellae in 1-3 seconds (Koce, 1999). Intervals between consecutive stimulations were 1.5 min. We recorded EOG signal using $Ag/AgCl_2$ electrode with Ringer-agar bridge positioned ~1mm above the olfactory epithelium. Depending on the EOG signal it was amplified 10-100× with a directly coupled amplifier (Grass P18D) and displayed on a pen recorder (OmniScribe recorder; Industrial Scientific, TX, USA). The amplitude of EOG was determined as a maximum height [mm] of a phasic displacement from a baseline.

EOG data analysis
To standardize EOG responses between different amino acids the response after stimulation with 10^{-4} M L-Ala was used as a standard. We compared the relative EOG amplitudes of different amino acids in intact (Koce; 1997) and regenerated olfactory organs using Pearson's correlation test. We determined the median of relative EOG amplitudes for each amino acid and calculated Pearson's correlation coefficient that was t-tested [R, $p<0.05$].

Behavioral tests in catfish with intact and regenerated olfactory organs
The first group of intact catfish were conditioned to L-alanine (L-Ala) (N=15) and the second group to L-valine (L-Val) (N=15). Amino acids solutions (0.1 M) were prepared fresh less than 30 minutes before the experiments. Using Pasteur pipettes suspended above the aerated area of the aquarium we delivered 2 milliliters of a stimulating solution into the aquarium water. Vertical turbulent currents driven by air-bubbles released down currents in the immediate vicinity of the bubbling area. The down current rapidly transported high concentration stimulus eddies to the bottom and across the entire aquarium reaching the head of the

catfish in less than 30 seconds (Valentincic and Caprio, 1994). During the 90 second experiments the catfish activity was video-recorded (MV1, Canon) and evaluated by counting number of turns greater than 90º. We rewarded the catfish 90 seconds after the conditioned amino acid delivery. In control experiments dechlorinated tap water was delivered into the aquarium. The number of turns during control, conditioning and test procedures were compared statistically using Wilcoxon sum of ranks test [$p<0.05$]; experiments conducted on the same day were compared statistically.
We investigated the catfish responses to amino acid stimuli in intact catfish and three months after the beginning of the olfactory organ regeneration. Catfish with regenerated olfactory organs were first conditioned to the same amino acids (L-Val or L-Ala) as prior the surgery and re-tested for discrimination of amino acids.

Amino acids used in behavioral test and EOG recordings
We used the highest purity (≥ 98%) amino acids purchased either from Sigma (Sigma Chemical, St.Luis, MO) or Fluka (Fluka Chemika-BioChemika, Switzerland): L-Ala, β-alanine (β-Ala), L-arginine hydrochloride (L-ArgHCl), glycine (Gly), L-threonine (L-Thr), L-leucine (L-Leu), L-serine (L-Ser), L-isoleucine (L-Ile), L-norleucine (L-nLeu), L-Val, L-histidine (L-His), L-norvaline (L-nVal), L-cysteine hydrochloride (L-Cys), L-asparagine (L-Asn), L-lysine hydrochloride (L-LysHCl) and L-methionine (L-Met). In the behavioral experiments we also tested eight binary mixtures of L-Val with another amino acid. L-Val was the more stimulatory component of the mixture in L-Val (30mM)/L-Lys (3.3mM), L-Val (30mM)/L-Arg (3.3mM), L-Val (30mM)/L-His (6.6mM), L-Val (30mM)/Gly (13.3mM) mixture and it was the less stimulatory component of the mixture in L-Cys (30mM)/L-Val (16.6mM), L-Ala (30mM)/L-Val (10mM), L-Asn (30mM)/L-Val (6.6mM), L-Ser (30mM)/L-Val (3.3mM) mixtures (Valentincic et al., 2000a).

RESULTS

Olfactory organ regeneration
Intact olfactory organs of black bullhead catfish (*Ameiurus melas*) consisted of 24-34 olfactory lamellae. Two months after their partial extirpation either small rosetae or fan-like olfactory organs regenerated (N= 90). The regenerated olfactory organs were considerably smaller than the intact organs. Each regenerated organ contained 13-22 olfactory lamellae. Some of the regenerated olfactory lamellae were fingerlike. Finger-like excrescences were in many cases located at the edge of the regenerated rosetae.

Connections between olfactory receptor neurons and the olfactory bulb
We retrogradely, from the olfactory bulb (OB) to the olfactory epithelium, labeled olfactory receptor neurons (ORNs) using fluorescent tracer DiI (intact olfactory organs, N=52; regenerated olfactory organs, N=17). In intact olfactory organs we observed axons of tall (ciliated) ORNs targeting anterior-ventral (N=18), posterior-ventral (N=4), anterior-dorsal (N=5) and lateral-dorsal (N=2) OB areas and intermediate (microvillar) ORNs targeting median-ventral (N=3), lateral-ventral (N=15), median-dorsal (N=2) and posterior-dorsal (N=3) OB areas. Crypt (short) cells were observed only on two olfactory organs. The somas of the crypt cells are ovoid; they are situated near the surface of the sensory epithelium. Somas with dendrites of the tall ciliated ORNs are bottle shaped. They reached the surface and the basal lamina of the sensory epithelium. Somas with dendrites of the intermediate microvillar ORNs do not reach the basal lamina. They occupy the second third of the sensory epithelium depth.
Like in intact in regenerated olfactory organs axons of tall (ciliated) ORNs projected to the anterior-ventral (N=9) OB area, whereas axons intermediate (microvillar) ORNs projected to the lateral-ventral (N=8) OB area.

Electroolfactogram (EOG)
We measured the EOG in response to amino acid stimulation of regenerated olfactory organs of the L-Ala and L-Val conditioned catfish 6 and 9 months after the partial excision of olfactory organs. In the catfish conditioned to L-Ala the amino acids L-Met and L-nVal evoked the largest EOG amplitudes. β-Ala evoked

the smallest EOG responses. In L-Val conditioned catfish the most stimulatory amino acids were L-Cys, L-Met, L-nLeu, L-Asn and L-nVal; the least stimulatory amino acids were Gly, L-Ile, L-Val and L –His.
We compared the stimulatory effectivenesses of amino acids in intact (Koce, 1997) and regenerated olfactory organs. For both, L-Ala and L-Val conditioned catfish the correlation of amino acid responses in intact and regenerated olfactory organs was high and significant (Pearson correlation coefficients, $R_{L\text{-}Ala}$=0.98 ($p<0.05$) and $R_{L\text{-}Val}$=0.93 ($p<0.05$)).

Olfactory discrimination of amino acids in catfish with intact and regenerated olfactory organs

L-Ala conditioned catfish
We tested olfactory discrimination of amino acids in the same bullhead catfish twice, before olfactory organs excision and after their excision and regeneration. Intact catfish were conditioned to L-Ala. The turning responses of the catfish was evaluated by counting number of turns above 90° in 90 seconds after the conditioning stimulus delivery into the experimental aquarium. During the first 20 conditioning trials with L-Ala the intact catfish turning response increased from median of 5 turns to medians 25-32 turns. Subsequently (trials 25-36) the conditioned response to L-Ala stabilized at 13 -30 turns during 90 second experiments. In cross tests we compared numbers of catfish turns after the delivery of the conditioning and non-conditioning stimulus solutions into the aquaria. Except L-Ser and Gly the intact catfish discriminated L-Ala from all other amino acids [$p<0.05$]. Additional discrimination training did not improve L-Ala/Gly and L-Ala/L-Ser discrimination. Intact catfish responded to L-Ala with up to six times greater number of turns than to amino acids L-Cys, L-Met, L-nVal, L-Thr, L-Arg and β-Ala. The partial excision of the olfactory rosetae was followed by the olfactory lamellae regeneration. After the morphological regeneration of the olfactory organs we presented the previous conditioning stimulus L-Ala to the same catfish repeatedly. Their turning responses to L-Ala were relatively large from the first trial on and they did not increase during further conditioning trials. With exception of the first test, these catfish discriminated L-Ala from all tested amino acids including L-Ser and Gly [$p<0.05$]. They responded to L-Ala at least three times more than to non-conditioned amino acid stimuli. The catfish responses to L-Ser and Gly were initially greater than the responses to other non-conditioned amino acids. During discrimination training the turning response to L-Ser and Gly decreased to the level of the non-conditioned stimuli.

L-Val conditioned catfish
During the conditioning to L-Val the catfish response initially increased from medians of 7-14 to medians of 29-41 turns. The conditioned response to L-Val stabilized after 40 conditioning sessions. With an exception of L-Ile the intact catfish discriminated the conditioned stimulus L-Val from all other amino acids [$p<0.05$]. They responded to L-Val at least twice as much as to other amino acid stimuli: Gly, L-Cys, L-Met, L-nVal, L-Thr, L-Asn, L-nLeu, L-Leu, L-Ser, L-His, L-LysHCl, L-ArgHCl and L-Ala. We also tested olfactory discrimination of L-Val from its binary mixtures with other amino acids. The intact catfish were unable to discriminate the L-Val from the binary mixtures containing L-Val as more stimulatory component [$p>0.05$], however they discriminated L-Val from binary mixtures with L-Val as the less stimulatory component [$p<0.05$].
After the morphological regeneration of the olfactory organs we presented the previous conditioning stimulus L-Val to the same catfish repeatedly. The catfish initial response to L-Val was greater than in the same intact catfish and it increased to medians of 21-32 turns after 11 trials. The catfish with the regenerated olfactory organs were tested for amino acid discrimination three months after the partial olfactory organ excision. Contrary to the intact animals these catfish initially did not discriminate conditioned stimulus L-Val from L-nLeu, L-Met and L-Ile [$p>0.05$], however; five months after the surgery they discriminated the conditioned stimulus from almost all other tested amino acids [$p<0.05$]. As the intact catfish, these animas were unable to discriminate L-Val from L-Ile [$p>0.05$]. In tests of olfactory discrimination of L-Val from its binary mixtures with other amino acids the catfish with regenerated olfactory rosetae did not discriminate the L-Val from the binary mixtures containing L-Val as the more stimulatory amino acid [$p>0.05$], however they discriminated L-Val from binary mixtures with L-Val as less stimulatory amino acid [$p<0.05$]. The catfish responded to L-Val and to binary mixtures with L-Val as more stimulatory component at least twice as much as to the mixtures containing L-Val as less stimulatory amino acid.

DISCUSSION

Partial excisions of catfish olfactory organs triggered regeneration of olfactory organs, small - fully functional olfactory rosetae emerged. The regenerated rosetae enabled olfactory discrimination of amino acids and responded to amino acids electrophysiologically. The small rosetae contained intermediate microvillar and tall ciliated ORNs that targeted the same OB areas as in intact olfactory organs.

Connections of the ORNs to the regenerated olfactory bulb

In fish different types of ORNs converge to different OB areas (Morita et al., 1996; Morita and Finger, 1998; Hansen et. al., 2003; Hansen et al.; 2005). The different types of ORNs respond to different classes of odorants including amino acids, nucleotides and bile salts (Hansen et. al., 2003; Hansen et al.; 2005; Nikonov and Caprio, 2001). In the black bullhead catfish tall ciliated ORNs converged to posterior ventral, anterior and lateral dorsal OB areas, whereas axons of intermediate microvillar ORNs targeted anterior dorsal, medial, posterior and lateral ventral OB areas. In the channel catfish the DiI crystals inserted into ventral and medial OB labeled the ciliated ORNs, whereas microvillar ORNs targeted almost exclusively the dorsal OB (Hansen et al., 2003). In the channel catfish tall ORNs responded to amino acids and bile salts, whereas intermediate microvillar ORNs responded to amino acids and nucleotides (Hansen et al., 2003; Nikonov and Caprio, 2001).

We inserted DiI crystals into the anterior and lateral ventral OB areas of the black bullhead catfish with regenerated olfactory rosetae. In the channel catfish and zebrafish these OB areas responded exclusively to amino acids (Nikonov and Caprio, 2001; Friedrich an Korsching, 1997). In the regenerated olfactory organs of black bullhead catfish the tall ciliated and intermediate microvillar ORNs targeted the same OB areas as in the intact olfactory organs. Tall ciliated ORNs were connected to anterior ventral OB area, whereas intermediate microvillar ORNs targeted lateral ventral OB area. Similar results were observed in mice after a chemical damage to the olfactory epithelium (Cummings et al., 2000; Sengoku et al.; 2001). In the regenerated olfactory organs of mice new ORNs axons re-connected to the same OB areas as in intact olfactory organ.

Electroolfactogram (EOG)

Amino acids' relative EOG amplitudes in regenerated olfactory organs were nearly equal to their relative EOG amplitudes in intact olfactory organs (Koce, 1997; Koce and Valentincic, 2000). The EOG amplitudes of highly stimulatory amino acids were large, whereas those of poorly stimulatory amino acids were small. EOG is hypothetically a sum of ORNs' receptor potentials (Ottoson, 1971; Caprio, 1978, 1980; Silver, 1982; Koce and Valentinčič, 2000; Scott and Scott-Johnson, 2002), which indicates that similar proportions of ORNs responded to specific amino acids in intact and regenerated olfactory rosetae. After recovery from transections of the olfactory nerves, EOG responses were reported for the regenerated olfactory organs of goldfish (Zippel et al., 1993), rainbow trout (Evans and Hara, 1985), frog (Troitskaia, 1996) and pigeon (Oley et al., 1975; Bedini et al., 1976).

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Intact catfish were conditioned either to L-Ala or to L-Val. During conditioning the duration of food search increased and the swimming become faster. Food searching behavior after stimulation with non-conditioned amino acids remained short and less intense. Counts of turns greater than 90° enabled the identification of amino acids that were perceived as equal to the conditioned stimulus (Valentincic et al., 2000a). Intact catfish conditioned to L-Ala could not discriminate L-Ala from L-Ser and Gly. These catfish responded to L-Ala stimulation with at least three times more intense swimming than to other amino acids. L-Val conditioned intact catfish discriminated L-Val from almost all other amino acids; however, they were unable to discriminate L-Ile from L-Val.

After the regeneration of the partially excised olfactory rosetae we re-conditioned the same catfish to either to L-Ala or to L-Val. During the re-conditioning to L-Ala the catfish response to L-Ala was large from the beginning and it did not increase during further conditioning. Since L-Ala is a highly stimulatory taste stimulus that releases swimming behavior (Caprio, 1975; Valentincic et al. 1994), the initial large response can not be assigned to olfactory stimulation. Surprisingly, in the L-Ala conditioned catfish, small regenerated olfactory rosetae enabled greater discrimination of amino acids than the intact olfactory rosetae. The catfish with regenerated olfactory rosetae discriminated L-Ala from all other amino acids including L-Ser and Gly. Differential olfactory discrimination abilities after the regeneration of olfactory rosetae were hypothetically a result of a differential gene expression during regeneration. In catfish with regenerated olfactory organs an initial large response to L-Val and its further increase occurred hypothetically due to a

reduced inhibition of feeding behavior in the catfish that spent long periods of time in the experimental aquaria - fear reduction was typical for such animals (Valentincic, 1994). Hamsters with regenerated olfactory organs also required a second discrimination training to reestablish the previous olfactory discrimination capabilities (Yee and Costanzo, 1995, 1998). After the 35 discrimination training trials the discriminatory abilities of the catfish with regenerated olfactory organs were the same as in the intact catfish. These catfish discriminated L-Val from almost all other amino acids; however, they were unable to discriminate L-Ile from L-Val.

We tested catfish with intact and regenerated olfactory organs for discrimination of L-Val from its binary mixtures with other amino acids. Bullhead catfish with intact and regenerated olfactory organs perceived the binary mixtures of amino acids as their more stimulatory components (Valentincic et al, 2000b). They discriminated L-Val from the binary mixtures containing L-Val as the less stimulatory component; however, they did not discriminate L-Val from the binary mixtures containing L-Val as the more stimulatory component.