Núcleo do Tracto Olfactivo Lateral
Estudo Morfológico, Histoquímico e Funcional

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“It always seems impossible until it's done.”

Nelson Mandela

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LISTA DE ABREVIATURAS

CB	calbindina
CR	calretinina
GABA	ácido gama-aminobutírico
nLOT	núcleo do tracto olfactivo lateral
nLOT1	camada 1 do núcleo do tracto olfactivo lateral
nLOT2	camada 2 do núcleo do tracto olfactivo lateral
nLOT3	camada 3 do núcleo do tracto olfactivo lateral
NPY	neuropeptídeo Y
PV	parvalbumina
VAChT	*vesicular acetylcholine transporter*
VIP	polipeptídeo vasoactivo intestinal

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INTRODUÇÃO

SOBRE A IMPORTÂNCIA DO SENTIDO DO OLFACTO

No Homem, a importância do olfacto é muitas vezes depreciada relativamente a outros sentidos como a visão ou a audição. Filogeneticamente mais antigo, este sentido singular caracteriza-se pela sua relação com as emoções. A descoberta revolucionária da família dos genes responsáveis pela expressão dos receptores olfactivos, que permitiu a atribuição do Prémio Nobel em Fisiologia ou Medicina a Linda Buck e a Richard Axel em 2004 (Buck e Axel, 1991), gerou um grande incremento na investigação do olfacto e realçou a importância desse sentido na vida humana e dos animais. Os estudos realizados sobre o olfacto na área das neurociências procuram elucidar a organização do mais críptico dos nossos sentidos, desde os processos ímpares de transdução à sua integração com a neurogénese e circuitos olfactivos.

O olfacto envolve a detecção e a interpretação de odores através de elementos de transdução que permitem a sua compreensão por várias regiões do cérebro, seja para recordar um evento memorável ou para antecipar uma situação de perigo. Classicamente, o olfacto é, para os mamíferos macrosmáticos, considerado crucial para a reprodução, maternidade, regulação neuroendócrina, respostas emocionais, agressividade e reconhecimento dos pares, dos predadores e das presas (Ennis et al., 2015). Na espécie humana, este sentido parece ter desempenhado papel importante no comportamento social, na selecção do *habitat* e na evolução da dieta, devido à integração de sinais olfactivos e gustativos que resultam na percepção do gosto (Zelano e Sobel, 2005).

Na qualidade de vida

O impacto dos distúrbios do olfacto na qualidade vida do Homem é cada vez mais conhecido e valorizado (Blomqvist et al., 2004; Hummel e Nordin, 2005; Holbrook e Leopold, 2006). Os doentes com hiposmia ou anosmia queixam-se frequentemente de alterações de humor e do apetite, de menor percepção da higiene pessoal e de limitações na confecção dos alimentos (Temmel et al., 2002; Stevenson, 2010; Doty e Kamath, 2014). A dificuldade na detecção de alimentos deteriorados pode atingir 75% dos doentes com hiposmia, e a segurança das pessoas pode estar em risco por serem incapazes de reconhecer, por exemplo, fugas de gás, incêndios, pesticidas ou outros vapores químicos (Miwa et al., 2001). Igualmente, para as pessoas cuja profissão depende da qualidade da

sua função olfactiva, como é o caso dos cozinheiros, bombeiros, canalizadores, enólogos e químicos, os distúrbios do olfacto podem ser extremamente incapacitantes (Croy et al., 2014a).

Nas doenças psiquiátricas

A disfunção olfactiva tem sido relacionada com várias doenças psiquiátricas, tais como distúrbios afectivos (Postolache et al., 2002; Swiecicki et al., 2009) e do humor (Pause et al., 2001; Lombion-Pouthier et al., 2006; Croy e Hummel, 2017), anorexia nervosa (Kopala et al., 1995; Roessner et al., 2005; Rapps et al., 2010), perturbação de pânico (Kopala e Good, 1996) e psicoses (Hudry et al., 2002; Moberg et al., 2003; Corcoran et al., 2005).

A relação da disfunção olfactiva com a depressão foi estabelecida no Rato, designadamente através de modelos experimentais de bulbectomia olfactiva. Este procedimento provoca alterações diversas em várias regiões cerebrais e, principalmente, no sistema límbico devido à interrupção das conexões entre o bolbo olfactivo e os diversos componentes deste sistema (Jesberger e Richardson, 1988; Kelly et al., 1997; Song e Leonard, 2005; Oral et al., 2013). Este tipo de lesão resulta num processo de reorganização do córtex límbico e de outras áreas corticais que podem ser responsáveis por alterações comportamentais (Kelly et al., 1997; Wierońska et al., 2001; Negoias et al., 2010; Huckins et al., 2013; Croy et al., 2014b), e dos níveis de dopamina (Masini et al., 2004) e serotonina (van der Stelt et al., 2005). Não surpreende, pois, que modelos de depressão em animais, frequentemente induzidos através de stresse social ou meios farmacológicos, possam também ser induzidos por lesões do bolbo olfactivo, tendo sido demonstrado que, nestas condições experimentais, as alterações comportamentais, neuroquímicas, neuroendócrinas e imunológicas são semelhantes às observadas em doentes deprimidos (Kelly et al., 1997; Song e Leonard, 2005; Oral et al., 2013).

A extrapolação dos resultados obtidos em modelos animais para Homem deve ser cautelosa. Porém, por um lado, sabe-se que os doentes com perda de olfacto (Temmel et al., 2002) ou anosmia congénita (Croy et al., 2012) têm maior probabilidade de apresentar sinais de depressão e, curiosamente, têm bolbos olfactivos atrofiados (Negoias et al., 2010). Por outro, é igualmente reconhecido que os sintomas depressivos se acompanham

de menor capacidade olfactiva. No entanto, a disfunção olfactiva deve ser interpretada como sintoma e não como marcador de depressão (Croy e Hummel, 2017).

Nas doenças neurodegenerativas

A perda de olfacto tem também sido associada a várias doenças neurodegenerativas, incluindo a doença de Alzheimer (Doty et al., 1987; Hawkes, 2003; Attems et al., 2014), a doença de Parkinson idiopática (Haehner et al., 2007; Berendse et al., 2011; Doty, 2012), a coreia de Huntington (Moberg et al., 1987; Mesholam et al., 1998), a síndrome alcoólica de Korsakoff (Jones et al., 1975; Mair et al., 1986), a esclerose múltipla (Silva et al., 2012) e a esclerose lateral amiotrófica (Barresi et al., 2012). Destas patologias, a doença de Alzheimer e a doença de Parkinson idiopática têm sido as mais estudadas.

A doença de Alzheimer é uma das causas mais frequentes de demência e o seu diagnóstico precoce é fundamental para que a intervenção médica e social, junto dos doentes e seus familiares, seja atempada (Barresi et al., 2012). A hiposmia está presente em cerca de 90% destes doentes (Attems et al., 2014), é um sintoma/sinal precoce do desenvolvimento da doença e tende a agravar-se com a sua evolução (Albers et al., 2006; Attems et al., 2014). É, ainda, um auxiliar importante no diagnóstico diferencial da doença de Alzheimer relativamente a outras formas de demência (Bahar-Fuchs et al., 2010; Barresi et al., 2012). Tem sido sugerido que a doença de Alzheimer possa ter início ao nível do córtex entorrinal, com subsequente envolvimento progressivo de outras áreas temporais adjacentes, incluindo o hipocampo (Attems et al., 2005; Barresi et al., 2012). O bolbo olfactivo também sofre alterações, tendo sido consistentemente demonstrada a deposição de tranças neurofibrilares e de placas neuríticas tanto no bolbo como no tracto olfactivo destes pacientes. Verificou-se também, através da aplicação de técnicas estereológicas, que o volume do bolbo olfactivo se encontra reduzido nestes doentes (Mundiñano et al., 2011) e, através de estudos de ressonância magnética nuclear e funcional, que existe correlação significativa entre o grau de atrofia do bolbo olfactivo e o declínio cognitivo dos pacientes (Thomann et al., 2009) e, também, entre este parâmetro e a gravidade da sua disfunção olfactiva (Wang et al., 2010).

Os mecanismos celulares ou moleculares da hiposmia associada à doença de Parkinson idiopática também não estão identificados (Albers et al., 2006; Berendse et al., 2011). No entanto, é possível que as alterações dos níveis de neurotransmissores, nomeadamente da

acetilcolina, dopamina, noradrenalina e serotonina, no sistema olfactivo sejam a causa da alteração do olfacto nestes doentes (Doty, 2012). Tal como na doença de Alzheimer, o grau de disfunção olfactiva correlaciona-se com a gravidade da doença (Doty, 2012). A ausência de disfunção olfactiva noutras doenças neurodegenerativas, tornam o olfacto num importante auxiliar no diagnóstico desta patologia (Albers et al., 2006; Hawkes, 2006).

No envelhecimento

A par das doenças neurodegenerativas, a disfunção olfactiva pode ser um sintoma precoce de degenerescência neuronal (Doty e Kamath, 2014; Attems et al., 2015). Compreende-se assim que a disfunção olfactiva seja comum na população idosa e que a sua prevalência e gravidade aumentem substancialmente com a idade (Lafreniere e Mann, 2009; Schubert et al., 2012; Doty e Kamath, 2014; Attems et al., 2015). Sabe-se, ainda, que nos indivíduos idosos a presença de disfunção olfactiva está associada ao aumento da taxa de mortalidade (Wilson et al., 2011; Gopinath et al., 2012). Existe relativamente pouca informação sobre os mecanismos celulares e moleculares subjacentes à perda de olfacto associada à senescência, sabendo-se, no entanto, que as alterações da neurogénese no epitélio olfactivo e na zona sub-ventricular não parecem ser suficientes para justificar as alterações comportamentais que ocorrem no envelhecimento normal (Mobley et al., 2014). Porém, há estudos que mostram existirem alterações estruturais, da organização sináptica e dos níveis de neurotransmissores tanto no neuroepitélio olfactivo e bolbo olfactivo como no tubérculo olfactivo, núcleo olfactivo anterior, córtex piriforme, amígdala e córtex entorrinal (Adjei et al., 2013; Mobley et al., 2014; Attems et al., 2015). Acresce que, em estudos de ressonância magnética funcional realizados em humanos se verificou que a estimulação olfactiva activa o córtex olfactivo primário e áreas olfactivas associadas tanto em indivíduos jovens como em idosos, embora nestes o grau de activação seja menor (Wang et al., 2005).

No stresse

O cérebro é um órgão fundamental na resposta e adaptação ao stresse físico e social, determinando o que é ameaçador e regulando as respostas fisiológicas e comportamentais (McEwen et al., 2015). A resposta do cérebro aos factores de stresse é um processo

complexo, envolvendo várias áreas cerebrais e múltiplos mediadores. Os estudos realizados no Homem são escassos, mas sabe-se que num estudo realizado em indivíduos adultos com sintomas de irritabilidade, fadiga e ansiedade atribuíveis ao stresse causado pelas suas profissões, existe deficiente activação do córtex cingulado anterior, avaliado através de ressonância magnética funcional, após exposição a diversos odores (Jovanovic et al., 2011). Há também relatos sobre a associação de odores a memórias de experiências emocionais traumáticas, como por exemplo distúrbios de stress pós-traumático (Vermetten e Bremmer, 2003; Dileo et al., 2008; Croy et al., 2010), corroborados por estudos funcionais que mostram haver activação de um circuito neural que conecta as áreas olfactivas com os centros cerebrais que processam a memória (Vermetten et al., 2007). Estudos realizados em veteranos de guerra que associavam certos odores com as suas experiências traumáticas revelaram que estes indivíduos têm alterações volumétricas no córtex olfactivo e, também, que o grau de atrofia se correlaciona negativamente com a intensidade dos sintomas desencadeados pela exposição a determinados odores (Cortese et al., 2015). Por fim, e não menos importante, foi descrita diminuição do volume do bolbo olfactivo em adultos vítimas de maus-tratos infantis (Croy et al., 2013).

ANATOMIA DO SISTEMA OLFACTIVO

Os seres vivos estão permanentemente expostos a milhares de odores, que tornam o Mundo que povoamos particularmente vívido e emocional. A percepção do odor e a sua representação cortical são importantes desafios na investigação do olfacto na área das neurociências (Gottfried, 2010). Os odores, e as suas múltiplas combinações, ligam-se aos receptores dos neurónios olfactivos. A codificação/identificação do odor deve-se aos diferentes padrões de activação colectiva dos neurónios olfactivos. Estes neurónios localizam-se no epitélio olfactivo e os seus axónios atravessam a lâmina crivosa para terminar no bolbo olfactivo, onde estabelecem sinapses com os dendritos das células mitrais e tufosas ao nível dos glomérulos do bolbo olfactivo.

O bolbo olfactivo é o primeiro local onde, devido a circuitos locais, ocorre o processamento da informação olfactiva prévia à projecção para as áreas corticais. Além dos dois principais grupos de células de projecção, as células mitrais e tufosas, existem importantes interneurónios, as células granulares e justaglomerulares (Ennis et al., 2015).

Os axónios das células mitrais e tufosas integram-se no tracto olfactivo lateral para inervar várias regiões corticais, designadamente o córtex piriforme, vários núcleos da amígdala (nomeadamente os núcleos amigdalóide cortical anterior, amigdalóide cortical posterolateral, amigdalóide cortical posteromedial, a parte posteroventral do núcleo amigdalóide medial e o núcleo do tracto olfactivo lateral) e o núcleo supraóptico (Petrovich et al., 2001; Igarashi et al., 2012; Mobley et al., 2014; Ennis et al., 2015). As projecções do bolbo olfactivo são essencialmente ipsilaterais, existindo apenas algumas conexões para o hemisfério contralateral, que se estabelecem através da comissura anterior (Shipley e Ennis, 1996). Uma das características importantes do sistema olfactivo é a modulação da informação olfactiva, que inicia o seu processamento no bolbo olfactivo mediado pelas projecções que recebe de áreas olfactivas e não olfactivas. As aferências neuromoduladoras ao bolbo olfactivo surgem de três sistemas: noradrenérgico, proveniente do *locus coeruleus*, acetilcolinérgico, com origem no núcleo da banda diagonal de Broca no prosencéfalo basal, e serotoninérgico, procedente dos núcleos da rafe (Ennis et al., 2015). O bolbo olfactivo recebe ainda aferências com origem no córtex piriforme, núcleo olfactivo anterior, amígdala, córtex entorrinal lateral e hipotálamo.

O córtex olfactivo primário é subdividido em três grandes regiões: (1) o córtex olfactivo medial, que inclui o *indusium griseum*, a *taenia tecta*, o córtex infra-límbico e o tubérculo olfactivo; (2) o núcleo olfactivo anterior e (3) o córtex olfactivo lateral, que compreende os córtices piriforme, peri-amigdalóide, transicional e entorrinal (Ennis et al., 2015). O núcleo olfactivo anterior está interposto entre o bolbo olfactivo principal e o córtex piriforme. As suas células são essencialmente piramidais e projectam-se de forma maciça para o bolbo olfactivo principal contralateral. Resultados obtidos com recurso a lesões deste núcleo sugerem que desempenha um papel importante na transferência inter-hemisférica da informação dos odores e memória (Yan et al., 2008). Outros trabalhos envolvendo este núcleo relacionam-no com a localização espacial dos odores (Kikuta et al., 2008; Yan et al., 2008). O córtex piriforme localiza-se posteriormente à sua extremidade caudal e é o principal componente do córtex olfactivo primário. O conjunto formado pelos córtices piriforme, peri-amigdalóide e entorrinal e o hipocampo foi designado por "rinencéfalo" pelos anatomistas clássicos. Contudo, estudos ulteriores revelaram que o bolbo olfactivo principal não inerva directamente o hipocampo. O córtex piriforme é uma estrutura tri-laminar descrita como estrutura cortical de associação. Os seus neurónios, quando activados após exposição a um determinado odor, não têm

distribuição espacial preferencial e, simultaneamente, exibem regiões receptoras descontínuas, o que contrasta com outras áreas sensitivas em que as células que respondem a determinado estímulo estão agrupadas e têm pouca variabilidade de resposta (Stettler e Axel, 2009). A representação dos odores no córtex piriforme deve-se às conexões entre os neurónios das suas subdivisões anterior e posterior (Haberly, 2001; Stettler e Axel, 2009). As conexões que este estabelece com o córtex orbitofrontal, entorrinal e a amígdala, entre outros, reforçam as suas características de córtex associativo. Estabelece ainda conexões com o hipocampo, tálamo, hipotálamo e cerebelo (Doty e Kamath, 2014). O córtex orbitofrontal é reconhecido como desempenhando papel fundamental na percepção do gosto, combinando a informação do paladar, textura e odor (Rolls e Grabenhorst, 2008). Não surpreende, portanto, que as lesões efectuadas nesta região conduzam a um distúrbio na identificação dos odores e gostos (Jones-Gotman e Zatorre, 1988).

Existe uma segunda estrutura olfactiva importante, o órgão vomeronasal. Nos mamíferos, este órgão localiza-se na base do septo nasal e detecta principalmente odores não-voláteis (Ennis et al., 2015). Os seus neurónios receptores projectam-se para o bolbo olfactivo acessório. Classicamente separada do sistema olfactivo principal, o sistema olfactivo acessório converge com o principal não só em termos dos comportamentos em que estão envolvidos, mas também na partilha de algumas das áreas para onde projectam (Keller et al., 2009; Martinez-Marcos, 2009). Neste aspecto, são de referir, para além da amígdala medial e estria terminal, áreas olfactivas clássicas tais como o núcleo do tracto olfactivo lateral, o núcleo amigdalóide cortical anterior, a área de transição entre a amígdala e o córtex, e áreas previamente consideradas como sendo exclusivamente receptoras de informação vomeronasal, como é o caso da amígdala anterior ventral, *bed nucleus of the accessory olfactory tract*, e o núcleo amigdalóide medial anteroventral (Mucignat-Caretta et al., 2012). Já numa perspectiva funcional, o sistema olfactivo principal está envolvido na detecção de substâncias à distância, desempenhando o sistema olfactivo acessório um papel mais específico na exploração activa dos estímulos relevantes (Martínez-García et al., 2009). Não obstante estas diferenças, é cada vez mais consensual que ambos os sistemas participam na construção de uma percepção sensorial unitária.

Núcleo do tracto olfactivo lateral

O núcleo do tracto olfactivo lateral (nLOT) é uma região pouco desenvolvida no encéfalo dos primatas em comparação com animais macrosmáticos, que possuem um nLOT claramente identificável. No que respeita ao encéfalo humano, existe na literatura uma marcada discrepância a propósito deste núcleo (para revisão, ver Yilmazer-Hanke, 2012). Alguns autores incluem o nLOT como um dos membros da região amigdalóide superficial "quasi-cortical", localizando-se na interface entre o núcleo amigdalóide cortical anterior e a parte superior e rostral do núcleo cortical ventral (para revisão, ver Yilmazer-Hanke, 2012). Contudo, é importante referir que, embora alguns autores identifiquem no encéfalo dos primatas a região definida anteriormente como o nLOT, esses mesmos autores negam a homologia com o nLOT dos animais macrosmáticos. Efectivamente, esses autores assumem que o nLOT humano sofreu completa regressão filogenética uma vez que, ao contrário dos animais macrosmáticos, não recebe aferências de um sistema olfactivo acessório (para revisão, ver Yilmazer-Hanke, 2012). Em primatas não humanos, o nLOT, embora de difícil identificação em secções coradas pelo método de Nissl, apresenta grande densidade de fibras colinérgicas, característica que o identifica e que é encontrada também no nLOT de mamíferos macrosmáticos, como é o caso do Rato. Também no que concerne à hodologia do nLOT dos primatas, existe na literatura pouca informação disponível. Os poucos dados disponíveis indicam que este núcleo recebe aferências de áreas corticais e do hipotálamo (Pitkänen e Amaral, 1998), e que inerva o córtex entorrinal "olfactivo" (para revisão, ver Yilmazer-Hanke, 2012).

No Rato, o nLOT é actualmente incluído no grupo dos núcleos corticais da amígdala palial (Remedios et al., 2007; Hirata et al., 2009), embora outros autores o tenham considerado um componente da porção corticobasolateral da amígdala (McDonald, 2003) ou mesmo um componente integral do sistema olfactivo principal (Swanson e Petrovich, 1998; Pitkänen et al., 2000). É visível na superfície basal dos hemisférios cerebrais, onde aparenta estar integrado no núcleo cortical anterior da amígdala, que o separa, lateralmente, do córtex piriforme e, posteriormente, dos restantes núcleos corticais da amígdala. Relaciona-se anteriormente com a área amigdalóide anterior, que se interpõe entre ele e o tubérculo olfactivo (Millhouse e Uemura-Sumi, 1985; Olucha-Bordonau et al., 2015).

A sua neurogénese foi recentemente estudada em detalhe e é singular. Para além de se desenvolver mais tarde que os demais componentes da amígdala (Müller e O'Rahilly, 2006), a sua camada 1 é proveniente do pálio ventral e as camadas 2 e 3 parecem ter origem ou no pálio dorsal (Remedios et al., 2007; Subramanian et al., 2009) ou no pálio lateral (Puelles et al., 2000; Gorski et al., 2002; Medina et al., 2004), enquanto que os restantes componentes da amígdala cortical são derivados do pálio lateral ou do ventral (Medina et al., 2004; Olucha-Bordonau et al., 2015).

Nos animais macrosmáticos, o nLOT tem estrutura tri-laminar (McDonald, 1983; Millhouse e Uemura-Sumi, 1985). A sua camada ventral contém poucos neurónios (camada plexiforme, camada 1 ou nLOT1), a camada dorsal tem forma triangular e possui as células de maiores dimensões (camada polimórfica, camada 3 ou nLOT 3), e a camada intermédia apresenta a maior densidade celular (camada piramidal, camada 2 ou nLOT2). As células que residem no nLOT são semelhantes às observadas noutros núcleos da amígdala e no córtex cerebral (McDonald, 1983) e as suas fibras de projecção convergem em direcção ao vértice do nLOT3 para formar o feixe comissural da estria terminal (McDonald, 1983; Millhouse e Uemura-Sumi, 1985). O nLOT encontra-se ligado bidireccionalmente ao bolbo olfactivo, córtex piriforme e amígdala basolateral, e tem projecções importantes para o estriado ventral, alcançando algumas das suas fibras os córtices pré-frontal e insular (Price, 1973; Luskin e Price, 1983; McDonald, 1991; Jolkkonen et al., 2001; Santiago e Shammah-Lagnado, 2004).

A morfologia das células que compõem o nLOT foi estudada detalhadamente por vários autores em material impregnado pelo método de Golgi (McDonald, 1983; Millhouse e Uemura-Sumi, 1985). Sabe-se assim que, no Rato, é constituído essencialmente por células piramidais uniformes, o que contrasta com a sua maior variabilidade no córtex piriforme (McDonald, 1983; Millhouse e Uemura-Sumi, 1985). Sob o ponto de vista neuroquímico, expressa intensamente metais pesados (por exemplo, zinco), o que o distingue das áreas adjacentes (Olucha-Bordonau et al., 2015), e evidencia intensa marcação para a acetilcolinesterase, com uma intensidade comparável à da amígdala basolateral, e para o vGLUT2, sobretudo na camada 2 (Olucha-Bordonau et al., 2015). Tem igualmente marcação para os receptores das orexinas 1 e 2 (Marcus et al., 2001). O núcleo possui poucos neurónios GABAérgicos, à semelhança da amígdala basolateral (McDonald e Augustine, 1993). Foram descritos igualmente escassos neurónios positivos para colecistocinina (Záborsky et al., 1985), parvalbumina (PV, principalmente nas

camadas 2 e 3), calbindina (CB, na camada 3) e calretinina (CR, essencialmente na camada 2; Kemppainen e Pitkänen, 2000).

As atribuições funcionais do nLOT nunca foram estudadas em detalhe, acreditando diversos autores, com base nas áreas alvo das suas projecções, que este núcleo estaria envolvido na regulação do comportamento alimentar (Luskin e Price, 1983; Santiago e Shammah-Lagnado, 2004).

OBJECTIVOS

Com o conjunto de trabalhos incluídos na presente dissertação teve-se como objectivo analisar:

1. As características morfológicas e neuroquímicas do nLOT no Rato adulto

2. As atribuições funcionais e comportamentais do nLOT

3. As repercussões do envelhecimento nas características morfológicas e neuroquímicas do nLOT

4. As repercussões do stresse crónico nas características morfológicas e neuroquímicas do nLOT

5. As repercussões do stresse crónico em comportamentos e funções em que o nLOT está implicado

TRABALHOS

I.

Age effects on the nucleus of the lateral olfactory tract of the rat

RESEARCH ARTICLE

Age Effects on the Nucleus of the Lateral Olfactory Tract of the Rat

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ABSTRACT

The olfactory deficits that occur during aging influence the quality of life and have been regarded as a risk factor for malnutrition in the elderly. The nucleus of the lateral olfactory tract (nLOT) is a cortical nucleus of the pallial amygdala that has been implicated in feeding behavior. Here we present quantitative data on the anatomy of the nLOT in the adult rat and on the effects of age on its structure and neurochemistry. Total neuron numbers, neuronal volumes, and volumes of layers 1–3 of the nLOT were estimated in adult and old male rats using stereological techniques. We also estimated the total number of interneurons expressing neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), and the numerical density of the nLOT cholinergic varicosities. Our data show that aging is associated with a reduction of the total neuron numbers in the nLOT, due to cell loss in layers 2 and 3. There were no age-related variations in neuronal volumes. Similarly, the volume of the nLOT was unchanged in aged rats, except in layer 3 where it was reduced. The numerical density of cholinergic varicosities was also unchanged in aged rats. Conversely, the total numbers of NPY- and VIP-immunoreactive neurons were reduced by 55% and 30%, respectively, in aged rats. These findings include the nLOT in the list of cortical olfactory structures susceptible to aging and raise the possibility that the age-related changes that occur in the nLOT might contribute for the decline in olfactory functions reported in normal aging. J. Comp. Neurol. 524:759–771, 2016.

INDEXING TERMS: olfaction; aging; nucleus of the lateral olfactory tract; acetylcholine; neuropeptide Y; vasoactive intestinal polypeptide

Olfactory dysfunction significantly affects well-being, health, and quality of life (reviewed in Croy et al., 2014). Its prevalence increases with age, being present in over 50% of individuals aged between 65 and 80 years (Doty and Kamath, 2014). In recent years, it has become increasingly evident that impairment of odor detection can be used as a marker of cognitive decline and Alzheimer's dementia (Attems et al., 2014), and is associated with increased risk of death (Wilson et al., 2011). In addition to nonneural causes, age-dependent olfactory deficits can be due to changes at different levels of odor detection and processing in the brain. Functional imaging studies have demonstrated age-related changes in the processing of olfactory information, as revealed by a reduction in odor-induced activation of central olfactory pathways (reviewed in Doty and Kamath, 2014). Investigations carried out in humans and experimental animals have demonstrated the presence of age-associated changes in the integrity of the olfactory epithelium and in the volume, cell numbers, neuron morphology, synaptic organization, or neurotransmitter levels of the olfactory bulb and of several components of the olfactory cortex or olfaction-related brain regions, namely, the anterior olfactory nucleus, olfactory tubercle, piriform cortex, amygdala, and entorhinal cortex (reviewed in Doty and Kamath, 2014; Mobley et al., 2014). Yet despite the intense research efforts towards understanding the structural basis of the age-related olfactory dysfunction, much

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remains to be elucidated, particularly in the field of non-pathological aging.

One of the important consequences of olfactory dysfunction in aged humans is appetite suppression, which results in weight loss, malnutrition, impaired immunity, and deterioration of medical conditions (reviewed in Seiberling and Conley, 2004; Hummel and Nordin, 2005; Boyce and Shone, 2006; Boesveldt et al., 2011). The nucleus of the lateral olfactory tract (nLOT) is a superficial nucleus of the pallial amygdala (Remedios et al., 2007; Hirata et al., 2009), also regarded as a component of the olfactory amygdala (Swanson and Petrovich, 1998; Pitkänen, 2000), that has been implicated in feeding behavior (Luskin and Price, 1983; Santiago and Shammah-Lagnado, 2004). It is bidirectionally connected with the olfactory bulb and piriform cortex, and sends strong projections to the basolateral amygdala and ventral striatum, with some fibers reaching the prefrontal and the insular cortices (Price, 1973; Luskin and Price, 1983; McDonald, 1991; Jolkkonen et al., 2001; Santiago and Shammah-Lagnado, 2004). Due to its anatomical connections, it has been suggested that the nLOT might play a role in voluntary, instrumental behaviors dependent on complex learning associations presumably related to the control of food intake (Petrovich et al., 1996; Cardinal et al., 2002; Santiago and Shammah-Lagnado, 2004).

The development (Remedios et al., 2007; Hirata et al., 2009; Subramanian et al., 2009), cytoarchitecture (Price, 1973; McDonald, 1983; Millhouse and Uemura-Sumi, 1985; Dziewiatkowski et al., 1992), hodology (Price, 1973; Luskin and Price, 1983; Santiago and Shammah-Lagnado, 2004; Igarashi et al., 2012), and neurochemistry (Millhouse and Uemura-Sumi, 1985, Hecker and Mesulam, 1994; Kemppainen and Pitkänen, 2000; Real et al., 2009; Bombardi, 2011) of the nLOT have been described in earlier investigations. However, there are no data available on its basic morphometric parameters in the rat, despite being particularly amenable to stereological studies due to its distinctive three-layered organization (Price, 1973; Millhouse and Uemura-Sumi, 1985). In addition, the nLOT has not been examined in any investigation of normal or pathological aging. Yet it is well known that aging is associated with cholinergic dysfunction and that acetylcholine is intimately involved in the modulation of olfactory function (reviewed in Fletcher and Chen, 2010; D'Souza and Vijayaraghavan, 2014). There is also evidence for the presence of cholinergic-GABAergic interactions in the nLOT (Nitecka and Frotscher, 1989) and for the modulatory action of acetylcholine on GABAergic neurons expressing neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) in several regions of the brain (Arroyo et al., 2014; Pereira et al., 2015). In the current study we addressed these subjects and investigated, using stereological methods, the total neuron numbers, neuronal volumes, layer volumes, and density of varicosities immunoreactive for vesicular acetylcholine transporter (VAChT) in the nLOT of adult and old male rats. Due to the recognized role of NPY as a powerful stimulant of food intake (Lin et al., 2004; Loh et al., 2015) and of VIP in odor detection performance (Miller et al., 2014), we also analyzed, using the same stereological methods, the influence of age on the expression of these peptides in the nLOT.

MATERIALS AND METHODS

Animals

The experiments were conducted in young adult (6-month-old; $n = 18$) and aged (24-month-old; $n = 18$) male Wistar rats obtained from the Institute for Molecular and Cell Biology (Porto, Portugal). Rats were maintained in standard housing conditions (temperature, 22°C; humidity, 50%; 12:12 hour reversed light-dark cycle, with lights on at 7 AM) with free access to solid diet and tap water. All experimental procedures were performed in accordance with European Communities Council Directive (2010/63/EU) of 22 September 2010 and Portuguese Act No. 129/92. All efforts were made to minimize the number of animals used and their discomfort and suffering.

Tissue preparation

Rats were anesthetized, between 2 and 4 PM, with a solution containing 1% sodium pentobarbital and 4% chloral hydrate in physiological saline (3 ml/kg, intraperitoneally, i.p.). Rats (adult, $n = 6$; aged, $n = 6$) used for the estimation of total neuron numbers, nLOT volume, and neuronal volumes were killed by transcardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (PB), at pH 7.2. The brains were removed from the skulls, coded, postfixed for 30 days in fresh fixative, transected in the coronal plane through the anterior border of the optic chiasm and the anterior limit of the mammillary bodies, and embedded in glycolmethacrylate, as described in detail elsewhere (West et al., 1991). The blocks were then serially sectioned in the coronal plane, at a nominal thickness of 40 μm, using a Jung Multicut microtome. Sections were mounted serially and stained with a modified Giemsa solution (West et al., 1991).

Rats (adult, $n = 6$; aged, $n = 6$) used for the immunocytochemical detection of NPY and VIP were perfused transcardially with 150 ml of 0.1 M PB, pH 7.6, for

TABLE 1.
Primary Antibodies Reagents

Antigen	Immunogen	Manufacturer, catalog number, research resource identifier	Dilution
Vasoactive intestinal polypeptide (VIP)	Manufactured rabbit polyclonal anti-vasoactive intestinal polypeptide	Produced by the Netherlands Institute for Neuroscience (Amsterdam), N/A, RRID:AB_2513212	1:2,500
Neuropeptide Y (NPY)	Synthetic rabbit polyclonal anti-neuropeptide Y	Bachem, Cat# T-4070, RRID:AB_518504	1:10,000
Vesicular acetylcholine transporter (VAChT)	Synthetic goat polyclonal anti-vesicular acetylcholine transporter	Millipore, Cat# AB 1578, RRID:AB_10000324	1:15,000

vascular rinse, followed by 250 ml of a fixative solution containing 4% paraformaldehyde in PB, at pH 7.6. The brains were removed from the skulls, coded, immersed for 1 hour in the same fixative, and maintained overnight in a solution of 10% sucrose in PB, at 4°C. After trimming the brains as described above, the right and left hemispheres were isolated and alternately allocated for NPY or VIP immunostaining. The blocks were placed on a vibratome and serially sectioned in the coronal plane, at 40 μm, through the nLOT. Sections from each hemisphere were alternately collected and processed either for immunohistochemistry or for Giemsa staining. Sections to be immunostained were washed twice in phosphate-buffered saline (PBS) and treated with 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidase. They were then incubated overnight at 4°C with the primary antibody against NPY (T-4070, Bachem, Merseyside, UK; 1:10,000) or VIP (kindly provided by Dr. Arja Sluiter, Netherlands Institute for Neuroscience, Amsterdam, Netherlands; 1:2,500). Biotinylated goat antirabbit antibody (Vector Laboratories, Burlingame, CA; 1:400) was used as the secondary antibody. Afterward, sections were treated with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector; 1:800) and incubated for 10 minutes in 0.05% diaminobenzidine (DAB; Sigma) to which H_2O_2 was added to a final concentration of 0.01%. Both the primary and the secondary antibodies were diluted in PBS to which 0.5% Triton X-100 was added. Between different incubations, sections were rinsed with the same solution of PBS for at least 15 minutes. Sections were mounted on gelatin-coated slides, air-dried, dehydrated in a series of ethanol solutions (50%, 70%, 90%, and 100%), cleared in xylol, and coverslipped using Histomount (National Diagnostics, Atlanta, GA). To prevent variability in staining, sections from adult and old rats were processed in parallel. The same procedure was followed for control sections, which were incubated without primary antisera; no immunostaining was observed in these sections (data not shown). Sections allocated for Giemsa staining were mounted on gelatin-coated slides, air-dried, stained with Giemsa (Merck, Darmstadt, Germany), dehydrated, and coverslipped with Histomount. These sections were used to help in the identification of the nLOT boundaries in immunostained sections.

Rats (adult, $n = 6$; aged, $n = 6$) used for VAChT immunostaining were anesthetized and perfused as described above. The brains were trimmed as described above and serially sectioned in the coronal plane, at 40 μm, through the nLOT. Because the nLOT is readily visible and its layers are easily defined in VAChT-immunostained material, only alternate sections were collected. All immunohistochemical steps were carried out as described above, with the following exceptions. After immersion in a 5% solution of rabbit normal serum (Vector) in PBS, for 30 minutes at room temperature, sections were incubated with the primary antiserum (AB1578, Chemicon, Millipore, Bedford, MA; 1:15,000) for 72 hours at 4°C. Biotinylated rabbit anti-goat antibody (Vector; 1:400) was used as the secondary antibody.

Antibody characterization

All antibodies used in this study are as cited in Table 1 and were previously used and tested as detailed below. The rabbit anti-VIP polyclonal antibody was produced by the Netherlands Institute for Neuroscience (Amsterdam, RRID:AB_2513212) and was tested for its specificity at the Institute (Romijn et al., 1998). Specificity controls of the antibody were carried out according to a described procedure (Dai et al., 1997; Goncharuk et al., 2001). This antibody has been widely used for characterization of VIP immunohistochemistry in the rat suprachiasmatic nucleus (Dai et al., 1997; Romijn et al., 1998; Madeira et al., 2004; Pereira et al., 2005) and medial prefrontal cortex (Pereira et al., 2015). The rabbit anti-NPY polyclonal antibody (Bachem, Cat. no. T-4070, RRID:AB_518504) was produced against the YPSKPDNPGEDAPA sequence of the rat neuropeptide Y protein, and it was demonstrated that antibody immunostaining is abolished by preincubation with synthetic NPY (Rowan et al., 1993). In a tetracycline-driven conditional knockout (KO) mouse, in

which levels of mRNA and protein were reduced, the antibody staining of the brain was comparably reduced at that timepoint (Ste Marie et al., 2005). The goat polyclonal anti-VAChT antibody (Millipore, Cat. no. AB 1578, RRID:AB_10000324) is a synthetic peptide corresponding to the C-terminus of cloned rat VAChT (CSPPGPFDGCEDDYNYYSRS). It has been evaluated for its ability to stain vesicular acetylcholine transporter in the rat brain and to recognize a 65–70 kDa band in western blotting, corresponding to VAChT protein (technical information of the manufacturer). The anti-VAChT antibody has been previously successfully used in immunohistochemical studies in the rat mesopontine tegmentum and spinal cord (Ito et al., 2007; Brischoux et al., 2008).

Stereological analyses

The delineation of the nLOT and of its layers in Giemsa-stained sections was done on the basis of cytoarchitectonic criteria that were described in detail in previous publications (Price, 1973; McDonald, 1983; Millhouse and Uemura-Sumi, 1985). Accordingly, layer 1 (nLOT1; plexiform layer) lies next to the pia, is lightly stained, and contains very few neurons; layer 2 (nLOT2; pyramidal layer) lies dorsally to layer 1, is disc-shaped and densely populated with cells; layer 3 (nLOT3; polymorph layer) lies dorsally to layer 2 and has a triangular shape (Fig. 1). The estimates of layer volumes, total neuron numbers, neuronal volumes, and numerical density of cholinergic varicosities were obtained separately for layers 1–3. Conversely, no discrimination between layers was done when estimating the total number of NPY- and VIP-immunoreactive neurons due to their relatively small number in the nLOT. Stereological estimations were all performed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage, and an object rotator (Olympus, Albertslund, Denmark). A computer fitted with a framegrabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor.

For the estimates of layer volumes and total neuron numbers, all Giemsa-stained sections in which the nLOT was visualized were used, whereas the estimates of mean neuronal volumes were obtained from alternate sections. Layer volumes were estimated by applying the principle of Cavalieri and point counting techniques (Gundersen and Jensen, 1987; Madeira et al., 1995, 1997), at a final magnification of 100× at the screen level, using a grid of test points in which the area per point was 9.2 μm^2. On average, 300 points were counted per animal in nLOT1 and nLOT2 and 140 in nLOT3; the coefficient of error (CE) was no more than 0.05 for each volume estimate. The volume of each layer was calculated from the total number of points that fell on that layer and the section thickness, and the volume of the nLOT was computed as the sum of the volumes of the individual layers. The total number of neurons in each layer was estimated by applying the optical fractionator method (West et al., 1991; Madeira et al., 1995, 1997). In each section, the fields of view were systematically sampled using step sizes, in the x and y axis, of 90 μm (nLOT1), 120 μm (nLOT2), or 60 μm (nLOT3); the disectors had a counting frame area of 4,953 μm^2 (nLOT1), 693 μm^2 (nLOT2), or 1,189 μm^2 (nLOT3), and a fixed depth of 10 μm. The average number of neuron counted was 190 (nLOT1) and 220 (nLOT2 and nLOT3), and the mean CE of the estimates was, on average, less than 0.08. The total number of nLOT neurons was computed as the sum of the total number of neurons in layers 1–3. Neuronal density (number/mm^3) was calculated for each animal by dividing the number of neurons actually counted by the volume of the sampling fraction. The mean somatic volume $\bar{V}_N$) of nLOT neurons was estimated by applying the optical rotator (Tandrup et al., 1997; Leal et al., 1998). Neurons used for measurements were selected with optical disectors, as described above. Measurements of intersections between the cell membrane and the spatial line grid were performed using a two-grid line and two focal planes per each cell. The mean CE of the estimates was 0.05. Neurons were counted and measured at final magnification of 2000× at the screen level using a NA 1.40 × 100 oil-immersion objective lens.

The total numbers of NPY- and VIP-immunoreactive neurons were estimated by using the same stereological method. In each section, the fields of view were systematically sampled using step sizes of 110 μm (x axis) and 80 μm (y axis); the disector had a counting frame area of 7,924 μm^2 at the tissue level and a fixed depth of 10 μm. Neurons were considered as staining positively for NPY or VIP when they had a brown-stained perikaryal cytoplasm outlining a relatively unstained nucleus. The average number of neurons counted was 22 in NPY-immunostained sections and 62 in VIP-immunostained sections. The mean CE of the estimates was 0.15 and 0.11, respectively.

For the estimation of the numerical density (number/mm^3) of VAChT-immunoreactive varicosities, sampling boxes were superimposed onto the video images at the mid-level of each layer and the number of varicosities was counted using the optical disector method (West et al., 1991; Madeira et al., 1995). In each section, two fields of view were analyzed per layer. The area of the counting frames of the sampling boxes was 99 μm^2 and the height of the disector was 5 μm. On average, 500 VAChT-immunoreactive varicosities were counted per animal.

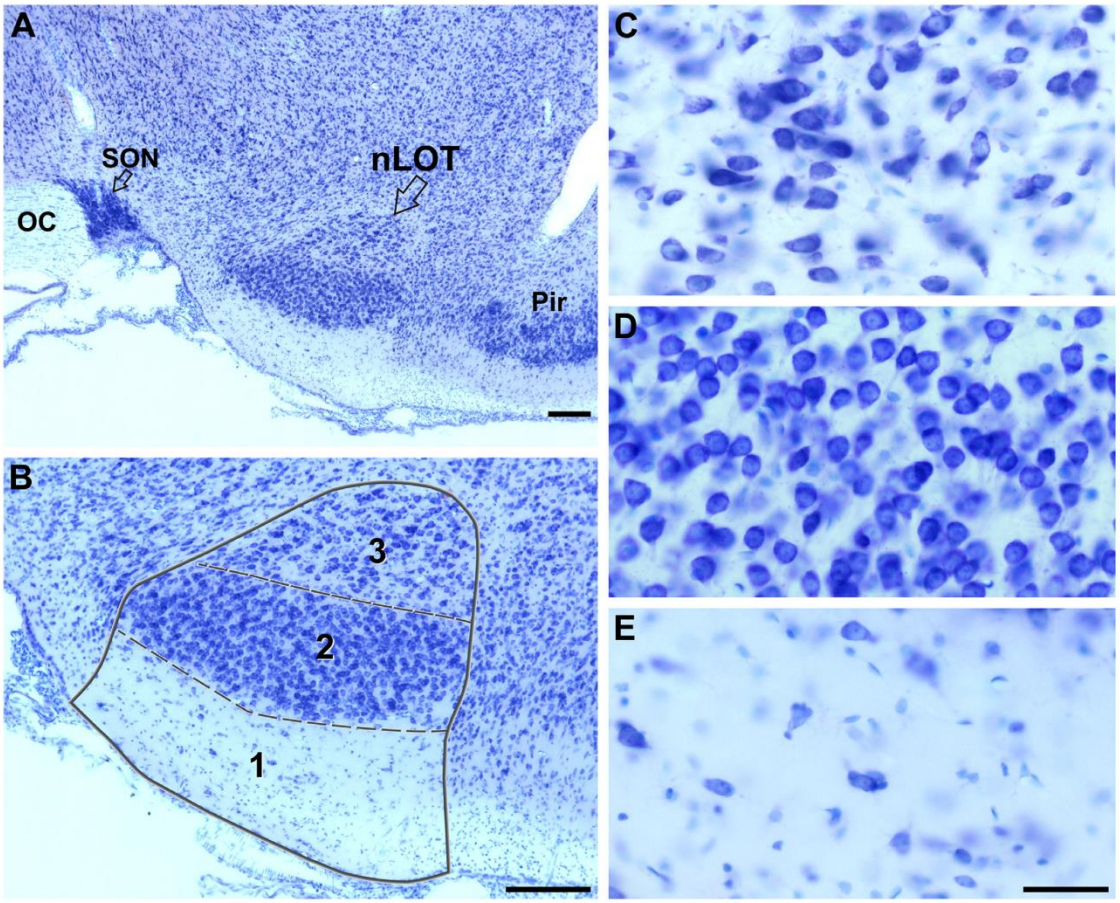

Figure 1. **A**: Low-power digital photomicrograph of a Giemsa-stained coronal section through the nLOT (arrow) of an adult male rat. Optic chiasm (OC). Piriform area (Pir). Supraoptic nucleus (SON). **B:** Higher magnification of the nLOT shown in A, where the cytoarchitectonic features are easily seen. The continuous line outlines the nLOT and the dashed lines mark the borders between adjacent layers. 1, Layer 1, more superficial and fibrous. 2, Layer 2, cell-dense layer. 3, Layer 3, loosely textured cell and fiber layer. Photomicrographs C–E show the magnified images of representative neurons of each layer of the nLOT shown in B. **C:** Layer 3 is characterized by its cell polymorphism and by the presence of the largest cells in the nLOT. **D:** Layer 2 is the most densely populated layer and is defined by its homogeneous pyramidal cells. **E:** Layer 1 contains very few neurons and is lightly stained. The brightness and contrast of the photomicrographs were equally adjusted using Adobe Photoshop 7.0. Scale bars = 200 μm in A,B; 50 μm in C–E. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical analyses

The CE of the estimates of neuron numbers and layer volumes was calculated as described by Gundersen et al. (1999). The precision of the individual estimates of mean somatic volumes was calculated as in Gundersen and Jensen (1987). A two-way analysis of variance (ANOVA) was used to test for main and interaction effects of age and layer on the mean neuronal volumes and on the density of VAChT-immunoreactive varicosities, followed by pairwise comparisons using the Tukey's HSD test. For the remaining data, age-related differences were separately assessed for each layer by means of a Student's *t*-test due to the large differences between layers in the magnitude of the parameters estimated. Differences were considered significant if $P < 0.05$. Data are presented as mean ± SD.

RESULTS

Layer volumes, total neuron numbers, and neuronal volumes

Our estimates show that, in adult rats, the nLOT has a volume of 0.24 mm^3 and contains 19,000 neurons (Table 2). The volumes of layers 1 and 2 are similar and

TABLE 2.
Morphometric Parameters Estimated in Giemsa-Stained Sections of the nLOT of Adult and Old rats

		Adult	Old	*t*-value
Volume (mm^3)	Layer 1	0.093 ± 0.008	0.098 ± 0.01	0.368
	Layer 2	0.099 ± 0.023	0.091 ± 0.007	0.456
	Layer 3	0.043 ± 0.004	0.037 ± 0.003	0.015
	nLOT	0.0235 ± 0.02	0.226 ± 0.018	0.444
Total neuron numbers	Layer 1	972 ± 139	939 ± 136	0.679
	Layer 2	15,551 ± 1,209	13,634 ± 749	0.009
	Layer 3	2,453 ± 242	1,747 ± 193	< 0.001
	nLOT	18,937 ± 1,165	16,320 ± 689	< 0.001
Neuronal density (number/10^{-3} mm^3)	Layer 1	11 ± 1	10 ± 0.5	0.119
	Layer 2	174 ± 7	146 ± 8	< 0.001
	Layer 3	61 ± 5	54 ± 5	0.03
Somatic volumes of neurons (μm^3)	Layer 1	516 ± 106	580 ± 67	(a)
	Layer 2	950 ± 66[1,2]	1,050 ± 96[1,2]	(a)
	Layer 3	788 ± 95[4]	806 ± 70[3]	(a)

Data are presented as mean ± SD. (a) Two-way ANOVA statistical analysis of neuron somatic volumes are presented in Table 3.
[1] $P < 0.05$ compared to layer 3
[2] $P < 0.001$ compared to layer 1
[3] $P < 0.01$
[4] $P < 0.001$ compared to layer 1.

approximately twice that of layer 3. However, 82% of the neurons are concentrated in layer 2, with layer 3 containing 13% and layer 1 only 5%. Therefore, the neuronal density was highest in layer 2, intermediate in layer 3, and lowest in layer 1.

The total volume of the nLOT did not differ between adult and aged rats. No age-related differences were also found in the volume of layers 1 and 2, but layer 3 was significantly smaller in aged than in adult rats (Table 2). The total number of neurons in the nLOT was significantly smaller (14%) in aged than in adult rats, as it was in layers 2 and 3 (12% and 29%, respectively), but not in layer 1. Identical age-related differences were found in the neuronal density of layers 2 and 3, but not of layer 1.

ANOVA tests revealed that the mean somatic volumes of nLOT neurons varied as a function of layer location and age (Tables 2 and 3). In adult as in aged rats, neurons were, on average, largest in layer 2, smallest in layer 1 and of intermediate size in layer 3. Despite the main effect of age on mean neuronal volumes, no significant differences between adult and aged rats were found in all layers when pairwise comparisons were conducted.

Total number of NPY- and VIP-immunoreactive neurons

In the adult rat, the nLOT contains, on average, 120 NPY-immunoreactive neurons and 300 VIP-immunoreactive neurons, which represents ~0.6% and 1.6%, respectively, of its neuronal population. While NPY-immunoreactive neurons could be seen scattered by layers 1–3, VIP-immunoreactive neurons were mostly concentrated in layer 2 (Figs. 2, 3).

In aged rats, the total number of NPY-immunoreactive neurons was reduced by 55%, and the total number of VIP-immunoreactive neurons by 30%, relative to those estimated in adult rats. Thus, in aged rats NPY- and VIP-immunoreactive neurons jointly represent 1.6% of the total number of neurons of the nLOT.

Numerical density of cholinergic varicosities

ANOVA tests revealed that the density of VAChT-immunoreactive varicosities varied as a function of layer localization (Table 3). In adult rats, the numerical density of the varicosities was significantly higher in layers 1 and 2 than in layer 3 (Fig. 4A,B). While the density of the varicosities was 12% higher in layer 2 than in layer 1, the difference was not statistically significant. Although ANOVA identified a significant effect of age on the numerical density of the varicosities (Table 3), post-hoc tests failed to detect significant differences between adult and aged rats in all nLOT layers (Fig. 4B).

DISCUSSION

In this study we provide detailed data on the basic quantitative features of the rat nLOT anatomy and direct evidence that aging is associated with changes in its structure and neurochemistry. In the adult rat, the nLOT has a volume of 0.24 mm^3 and contains 19,000 neurons. Most (82%) of the neurons are concentrated in nLOT2, whereas 13% are located in nLOT3 and 5% in nLOT1. Because layer 3 has half the volume of layers 1 and 2, which display identical volumes, the neuronal density is highest in layer 2, lowest in layer 1, and has intermediate values in layer 3, as is apparent in the

TABLE 3.
Results of Two-Way ANOVA Examining the Effects of Layer and Age on Mean Neuron Somatic Volumes and Numerical Density of Cholinergic Varicosities in the nLOT

	Layer		Age		Layer × Age	
	$F_{(2,30)}$	*P*	$F_{(1,30)}$	*P*	$F_{(2,30)}$	*P*
Neuron somatic volume	85.52	< 0.001	4.59	0.040	0.70	0.505
Numerical density of cholinergic varicosities	35.20	< 0.001	6.43	0.017	1.08	0.354

microscopic images of the nLOT and has been described in earlier studies (Price, 1973; McDonald, 1983; Millhouse and Uemura-Sumi, 1985). Interestingly, our estimates of total neuron numbers in the nLOT of the adult rat are globally smaller than those reported for the guinea-pig (Rowniak et al., 2005) and rabbit (Rowniak et al., 2007), and the volume of the nLOT is not comparable because different embedding media were employed. It is interesting to note that despite the different methods used for estimating neuronal volumes, our estimates are broadly similar to those reported by Milhouse and Uemura-Sumi (1985). The finding in our study that neurons of layer 2 are, on average, the largest neurons of the nLOT is not necessarily at odds with the observations of these authors that the largest cell bodies are located in layer 3 because, by using the disector as the tool for selecting neurons, our estimates represent number-weighted, and not volume-weighted, mean volumes.

In agreement with recent published data (Real et al., 2009), very few NPY-immunoreactive neurons were found in the nLOT of the adult rat and their cell bodies could be seen dispersed by layers 1–3. VIP-immunoreactive neurons are about three times more numerous than NPY-immunoreactive neurons and are preferentially concentrated in nLOT2. Together, they represent 2.2% of the total neuronal population of the adult nLOT. Assuming that the fraction of GABAergic neurons in the adult nLOT is similar to the 15–20% estimated in the basolateral amygdala (McDonald, 1983; McDonald and Augustine, 1992), to which the nLOT closely resembles (reviewed in Sah et al., 2004), NPY- and VIP-expressing neurons would represent 11–15% of all inhibitory interneurons in the nLOT of the adult rat. Finally, data obtained in the adult rat also show that the density of the cholinergic varicosities is lower in layer 3 than in layers 1 and 2. Yet, and despite the trend for the presence of higher densities in layer 2 than in layer 1, which is in line with earlier qualitative descriptions of the cholinergic innervation of the nLOT (Millhouse and Uemura-Sumi, 1985; Nitecka and Frotscher, 1989), the differences between these layers were not statistically significant.

It was also found that aging has no apparent effect on the volume of the nLOT. However, when the analyses were narrowed to focus on individual layers, we found that aging leads to a significant reduction in the volume of layer 3 without changing that of layers 1 and 2. Conversely, aging was associated with a 14% reduction in the total number of nLOT neurons due to cell loss in layers 2 and 3. These results are particularly interesting as they include the nLOT in the list of olfactory regions affected by aging (Doty and Kamath, 2014; Mobley et al., 2014) and oppose to the general belief that there is no overt cell loss during nonpathological aging (reviewed in Schliebs and Arendt, 2011). Layers 1–3 of the nLOT differ not only in the morphological type of their constituent neurons (Price, 1973; McDonald, 1983; Millhouse and Uemura-Sumi, 1985), but also in the neural circuits in which they are involved (reviewed in Santiago and Shammah-Lagnado, 2004). Specifically, nLOT1 and nLOT2 are the main recipients of afferents from the olfactory bulb, olfactory tubercle, and piriform cortex, and neurons in nLOT2 either project back to these regions or extensively innervate the shell of the nucleus accumbens. Somehow differently, nLOT3 is scarcely innervated by the olfactory bulb and strongly projects to the core of the nucleus accumbens, ventromedial part of the caudate-putamen, dorsal agranular insular cortex, and basolateral amygdala. Although studies on the structural effects of aging on these brain regions are few, the available information seems to indicate that neuronal vulnerability to aging does not markedly differ among the components of the neural circuits in which layers 1–3 participate. In fact, even though the number of neurons and interneurons in the olfactory bulb does not seem to be affected by aging in humans and experimental animals, there are reports of reductions in the length of dendritic trees and in the number of synapses in aged rodents (reviewed in Doty and Kamath, 2014; Mobley et al., 2014). Also, studies in the human amygdala have reported either age-related volume atrophy (Mu et al., 1999; Walhovd et al., 2005) or stability (Good et al., 2001; Jernigan et al., 2001; Grieve et al., 2005), whereas studies in rodents have shown that aging is associated with an increase in

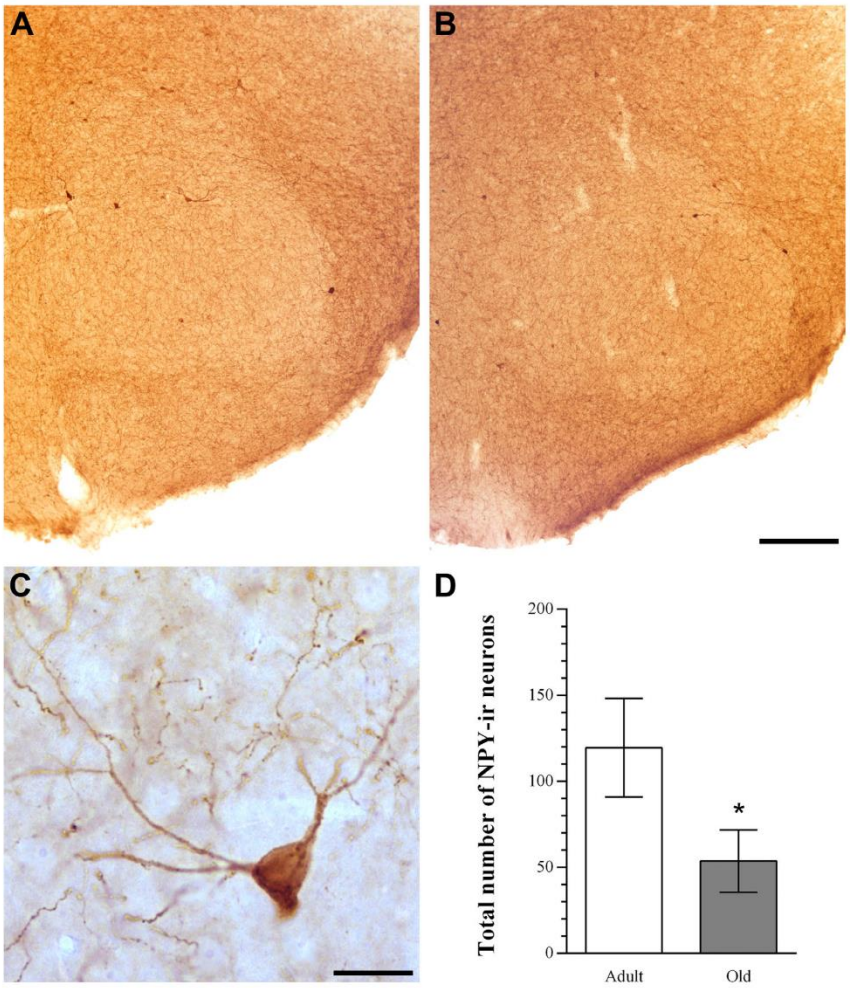

Figure 2. Representative photomicrographs of coronal sections of the nLOT immunostained for NPY of an adult (**A**) and an aged (**B**) rat. The number of neurons is higher in the adult than in the aged rat. **C:** High magnification of an nLOT neuron immunoreactive for NPY. The brightness and contrast of the photomicrographs were equally adjusted using Adobe Photoshop 7.0. **D:** Effects of age on the total number of NPY-immunoreactive neurons in the nLOT. Columns represent means and vertical bars ± 1 SD. $*P < 0.001$. Scale bars = 200 μm in A,B; 20 μm in C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the amygdala volume, unchanged total numbers, decreased neuronal densities, and enlarged dendritic arborizations (von Bohlen und Halbach and Unsicker, 2002; Rubinow et al., 2009; Rubinow and Juraska, 2009). In addition, a very recent study revealed a significant age-related reduction in the volume, number, and location of the mouse islands of Calleja (Adjei et al., 2013), to which the nLOT projects bilaterally (Luskin and Price, 1983; Santiago and Shammah-Lagnado, 2004).

The neuronal loss found in layers 2 and 3 of the nLOT was associated with a significant reduction in the total numbers of NPY- and VIP-immunoreactive cells. However, the number of NPY- and VIP-immunoreactive neurons lost during the process of aging represents just a relatively small fraction of the global cell loss that occurred in the nLOT. This indicates that, in addition to NPY-and VIP-producing neurons, other subpopulations of GABAergic and/or pyramidal neurons also undergo degeneration. The finding that the reduction in the total number of neurons was 2.5 times lower in layer 2 than in layer 3 suggests that pyramidal cells are less vulnerable to aging than GABAergic neurons. Supporting this view is our own observation that, in aged rats, NPY- and VIP-producing neurons jointly represent a smaller fraction of the total cell population of the nLOT than in adult rats (1.6% vs. 2.2%). Studies in the

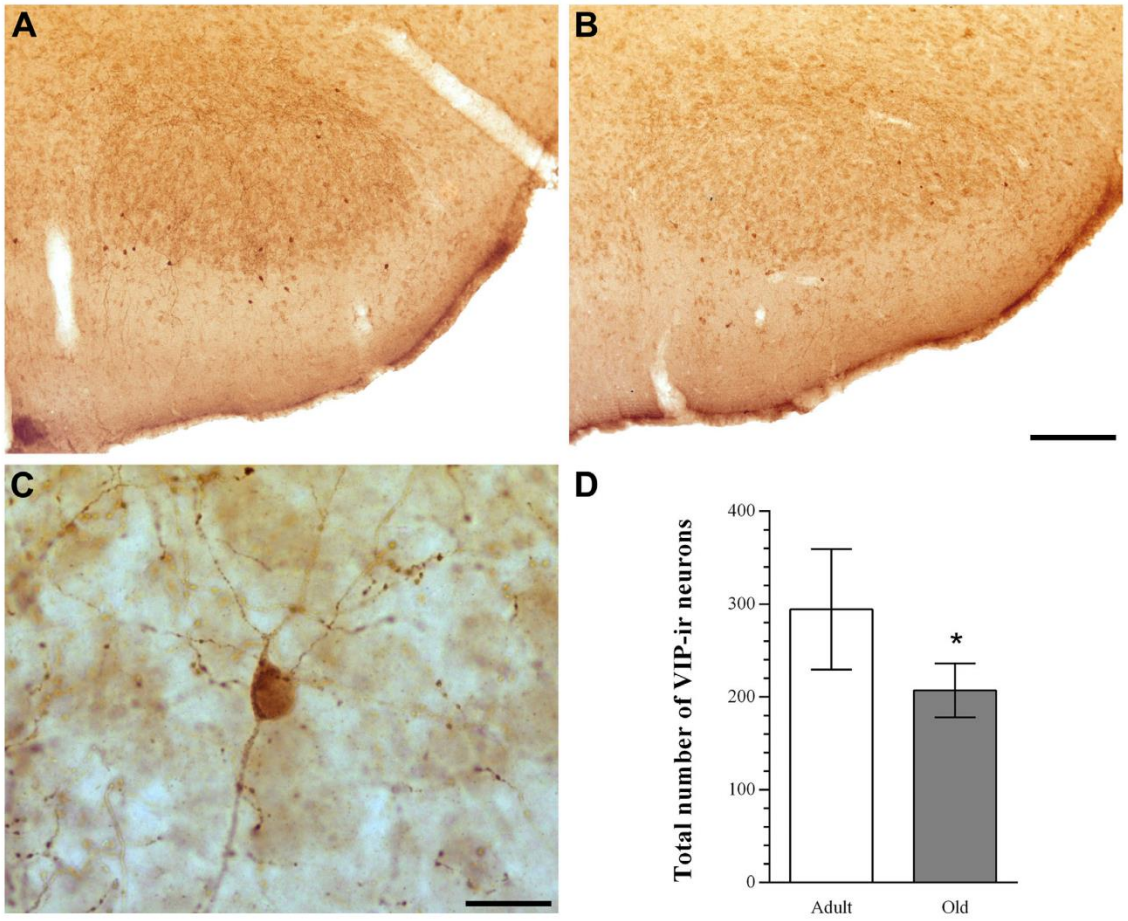

Figure 3. Representative photomicrographs of coronal sections of the nLOT immunostained for VIP of an adult (**A**) and an aged (**B**) rat. The number of neurons is higher in the adult than in the aged rat. **C:** High magnification of an nLOT neuron immunoreactive for VIP. The brightness and contrast of the photomicrographs were equally adjusted using Adobe Photoshop 7.0. **D:** Effects of age on the total number of VIP-immunoreactive neurons in the nLOT. Columns represent means and vertical bars ± 1 SD. $^*P < 0.05$. Scale bars = 200 μm in A,B; 20 μm in C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

basolateral amygdala indicate that interneurons innervate other local interneurons and tightly regulate the excitability of pyramidal cells, blocking their activity (Muller et al., 2007; for review see Giesbrecht et al., 2010; Spampanato et al., 2011). Thus, given similarities between the basolateral amygdala and the nLOT, one might argue that the age-related cell loss detected in the nLOT might alter the balance between excitatory glutamatergic and inhibitory GABAergic influences within the nLOT and, consequently, affect the activity of neural networks in which it is involved. In fact, there is evidence from studies in humans and rodents that aging is associated with reduced expression of NPY, VIP, somatostatin, orexin/hypocretin, and/or their receptors in brain regions that are direct or indirect targets of the nLOT, namely, the anterior olfactory nucleus, piriform cortex, amygdala, nucleus accumbens, and lateral hypothalamus (Hwang et al., 2001; Joo et al., 2005; Kessler et al., 2011; Pereira et al., 2013; Hunt et al., 2015). These changes might be relevant to the age-related deterioration of functions and behaviors in which NPY, VIP, and orexins have been implicated, such as the regulation of olfactory functions, food consumption, energy homeostasis, and modulation of circadian rhythms in the olfactory system (Lin et al., 2004; Burdakov et al., 2013; Miller et al., 2014; Loh et al., 2015; Nixon et al., 2015).

Acetylcholine is one of the most important neuromodulators in the brain, and the progressing memory deficits that occur during aging have been associated with

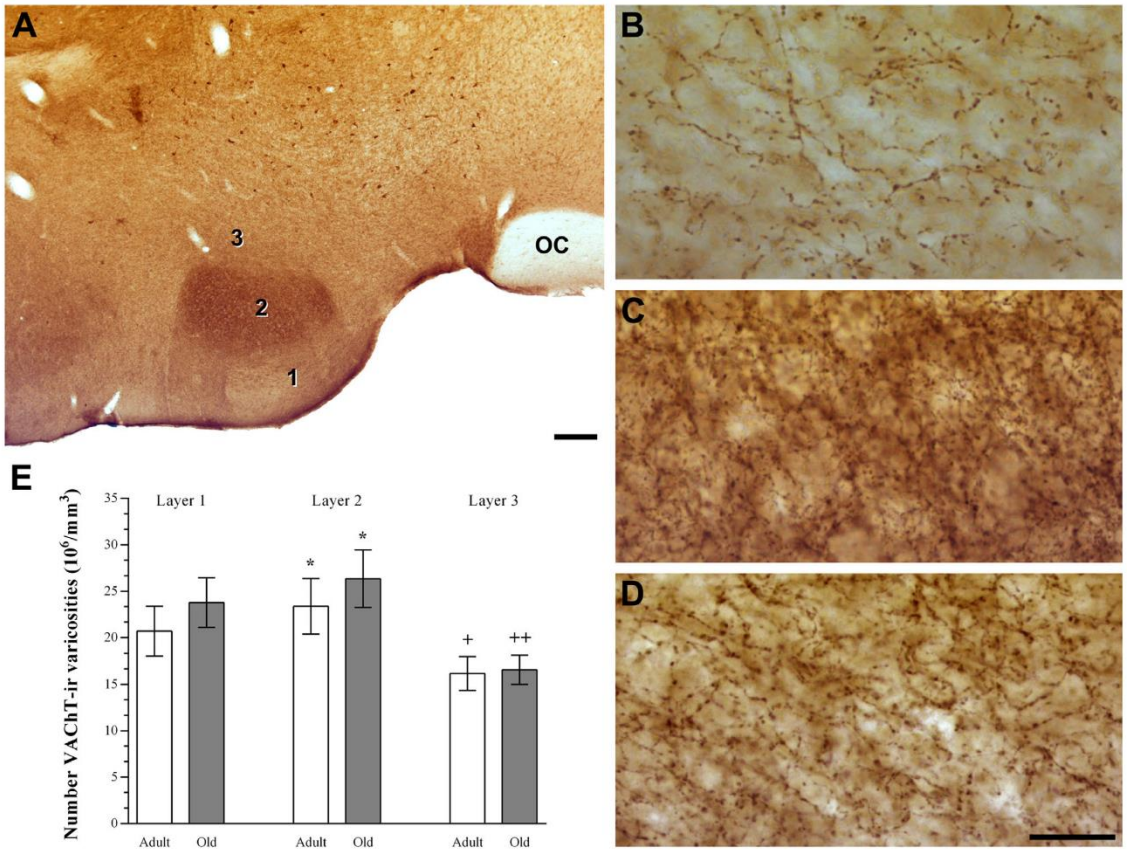

Figure 4. **A**: Photomicrographs of coronal sections immunostained for VAChT taken at approximately the mid-level of the nLOT of an adult rat. Optic chiasm (OC). B–D: higher magnification of photomicrograph A to illustrate the difference of density of cholinergic varicosities in layer 3 (**B**), layer 2 (**C**), and layer 1 (**D**). The brightness and contrast of the photomicrographs were equally adjusted using Adobe Photoshop 7.0. **E:** Graphic representation of the numerical density of cholinergic varicosities in layers 1, 2, and 3 of adult and old rats. The numerical density of the varicosities was significantly higher in layers 1 and 2 than in layer 3. $^{*}P < 0.001$ compared to layer 3; $^{+}P < 0.05$, $^{++}P < 0.001$ compared to layer 1. Columns represent means and vertical bars ± 1 SD. Scale bars = 200 μm in A; 20 μm in B–D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

degenerative changes in cholinergic neurons, with consequent cholinergic hypofunction (reviewed in Schliebs and Arendt, 2011). Because the nLOT is densely innervated by cholinergic fibers that establish synaptic contacts with GABAergic neurons (Nitecka and Frotscher, 1989), we analyzed the influence of age on the density of VAChT-immunoreactive varicosities in the nLOT. Our data show that the age-related cell loss in the nLOT is not consequent to changes in its cholinergic innervation, as we found no differences in the density of the cholinergic varicosities of layers 1–3 between adult and aged rats. However, this does not preclude the possibility that cholinergic neurotransmission might be affected in the nLOT of aged rats, as we have not analyzed, and there is no data available in the literature, on possible age-related variations in the expression of cholinergic receptors in the nLOT. It should be mentioned at this point that our results are in agreement with data from an earlier investigation in the human amygdala, where no age-related changes in density of cholinergic axons in the basolateral and central nuclei were found (Emre et al., 1993). It has been suggested and demonstrated that one of the factors that may contribute to the age-dependent degenerative changes in cholinergic neurons is the loss of trophic support (reviewed in Niewiadomska et al., 2009; see also Cardoso et al., 2006). It is therefore possible that the absence of age-related changes in the cholinergic innervation of the nLOT might be related to the fact that it arises from neurons located in the substantia innominata and ventral pallidum (Carlsen et al., 1985; Savander et al., 1995; McDonald et al., 2012), a subset of neurons of the

nucleus basalis magnocellularis that does not express nerve growth factor receptors (Hecker and Mesulam, 1994). It is also interesting to note that a subpopulation of nonpyramidal, presumably GABAergic, neurons of the nLOT and basolateral amygdala, namely, those expressing NPY, project back to the substantia innominata and ventral pallidum (McDonald et al., 2012). Therefore, it is tempting to suggest that the age-related changes that occur in the nLOT might also contribute to the apparent integrity of this subpopulation of cholinergic neurons and, consequently, of the cholinergic innervation of the nLOT in aged rats.

CONCLUSION

Data from the present study demonstrate the vulnerability of the nLOT to aging and suggest that this nucleus might contribute to the age-related functional deterioration of the olfactory system. They also show that the cell loss that occurs in the nLOT during aging is associated with a decrease in the number of neurons expressing NPY and VIP and, consequently, of the inhibitory signaling within the nLOT. Future studies to explore age-related mechanisms of neuronal loss and decreased expression of NPY and VIP in nLOT, and the impact of these changes in olfactory perception and feeding behaviors, will be important for understanding the neurobiological basis of olfactory deficits in normal aging and neurodegenerative disorders. Although the exact role of nLOT in olfactory processing remains unclear, studies focusing on modulating the structure of this nucleus will be critical in providing evidence that the degeneration of this nucleus is a necessary component of age and plausibly even neurodegenerative-dependent olfactory loss.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RPV, MDM. Acquisition of data: RPV, PAP. Analysis and interpretation of data: RPV, PAP, MDM. Drafting of the article: RPV, MDM. Study supervision: MDM.

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II.

The integrity of the nucleus of the lateral olfactory tract is essential for the normal functioning of the olfactory system

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ORIGINAL ARTICLE

The integrity of the nucleus of the lateral olfactory tract is essential for the normal functioning of the olfactory system

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Abstract The nucleus of the lateral olfactory tract (nLOT) is a relatively small component of the cortical pallial amygdala, with peculiar neurogenic, neurochemical and connectivity patterns. Although it has been suggested that it might be involved in non-pheromonal olfactory-guided behaviors, particularly feeding, the functional implications of the nLOT have never been investigated. In view of this fact, we have tackled this subject by performing a series of behavioral tests and by quantifying biological and biochemical parameters in sexually naïve adult male rats that were submitted to bilateral excitotoxic lesions of the nLOT. nLOT-lesioned rats had severe olfactory deficits with inability to detect and discriminate between odors. Additionally, they did not display innate behavioral responses to biologically relevant chemosignals. Specifically, nLOT-lesioned rats did not show avoidance towards predator odors or aggressive behaviors towards intruders, and had severely impaired sexual behavior. In fact, nLOT lesions abolished preference for odors of receptive females, reduced chemoinvestigatory behavior and eliminated mounting behavior. nLOT-lesioned rats had normal circulating levels of testosterone, did not display anxiety- or depressive-like behaviors, and had unimpaired cognitive functions and fear acquisition and memory. Altogether, our results suggest that the nLOT integrity is required for the normal functioning of the olfactory system.

Keywords Aggression · Anosmia · Attractive and avoidance behaviors · Cortical pallial amygdala · Olfactory cortex · Sexual behavior

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Introduction

The nucleus of the lateral olfactory tract (nLOT) is a small three-layered structure, with a volume of 0.24 mm^3 and 19,000 neurons (Vaz et al. 2016). It is located in the ventral surface of the brain interposed between the anterior amygdaloid area and the anterior medial amygdala (Ennis et al. 2015), and is related laterally with the anterior cortical amygdaloid nucleus (de Olmos et al. 2004). Because it has a cortical structure and receives a direct projection from the main olfactory bulb, some authors have regarded it as a component of the olfactory cortex (Price 1973; Swanson and Petrovich 1998). Due to its topographical relationships, other authors have considered it as one component of the olfactory amygdala, which also includes the anterior amygdaloid area, the anterior and the posterior cortical amygdaloid nuclei and the amygdalo-piriform transition area (reviewed in de Olmos et al. 2004). According to contemporary classification of amygdala nuclei (reviewed in Olucha-Bordonau et al. 2015), which is based on the conjoint analysis of morphological and neurochemical data, connectivity and pattern of gene expression during brain development, the nLOT has been included in the cortical pallial amygdala. It shares this classification with the anterior, the posteromedial and the posterolateral cortical nuclei, the bed nucleus of the

accessory olfactory tract, and the cortico-amygdala and amygdalo-piriform transition areas. However, the nLOT does not fit completely within this group due to its atypical features of development. First, it develops later than the remaining components of the amygdala (Müller and O'Rahilly 2006). Second, it has a mixed origin with its layer 1 originating from the ventral pallium and layers 2 and 3 possibly from the dorsal pallium (Remedios et al. 2007; Subramanian et al. 2009) or the lateral pallium (Puelles et al. 2000; Gorski et al. 2002; Medina et al. 2004), whereas the remaining components of the cortical pallial amygdala are derivatives either from the lateral or the ventral pallium (Medina et al. 2004; Olucha-Bordonau et al. 2015).

Along with its peculiar neurogenic pattern, the nLOT also stands up as a unique region within the group of the cortical pallial nuclei due to some particular aspects of its morphology, neurochemistry and connections. It displays a distinctive three-layered organization (Price 1973; McDonald 1983; Millhouse and Uemura-Sumi 1985; Vaz et al. 2016) and its layer 2 neurons, which are relatively large and represent over 80% of the total neuronal population of the nLOT (Vaz et al. 2016), express a type of vesicular glutamate transporter (type 2) that is considerably less abundant, or even non-existent, in other areas of the pallial amygdala (Hur and Zaborszky 2005). It has a much higher density of heavy metals (Friedman and Price 1984) and stains more intensely for cholinergic markers (Millhouse and Uemura-Sumi 1985; Vaz et al. 2016) than the surrounding structures. Unlike other components of the olfactory amygdala (Kevetter and Winans 1981; Swanson and Petrovich 1998; McDonald 2003; Gutiérrez-Castellanos et al. 2014), it does not send direct projections to the extended amygdala or the hypothalamus (Santiago and Shammah-Lagnado 2004). Instead, it is bi-directionally connected with the olfactory bulb and the piriform cortex and strongly innervates the basolateral amygdala and ventral striatum, with some fibers being conveyed to the prefrontal and insular cortices (Price 1973; Luskin and Price 1983; McDonald 1991; Jolkkonen et al. 2001; Santiago and Shammah-Lagnado 2004). Due to these connections, the nLOT has been thought of as being involved in non-pheromonal olfactory-guided behaviors, especially feeding (Petrovich et al. 1996; Cardinal et al. 2002; Santiago and Shammah-Lagnado 2004). However, no study has so far attempted to examine in detail the functional significance of the nLOT.

To begin to address this issue, we conducted a battery of behavioral tests in adult male rats submitted to excitotoxic lesions of the nLOT and compared the data with those obtained in sham-lesioned and control rats. Because, in addition to receiving afferents from the main olfactory bulb, the nLOT also receives direct and limited indirect inputs—via the posteromedial cortical amygdaloid nucleus—from the accessory olfactory bulb (Pro-Sistiaga et al. 2007; Gutiérrez-Castellanos et al. 2014), we have assessed its involvement in olfaction, innate reproductive (e.g., mating) and defensive (e.g., predator avoidance and aggression) behaviors, and also in sensorimotor, anxiety- and depression-like behaviors, fear conditioning, learning and memory.

Materials and methods

Animals

Sexually naïve male Wistar rats, derived from the Institute for Molecular and Cell Biology (Porto, Portugal), were used. After acclimation to laboratory conditions for at least 1 week, rats were single-housed and maintained in standard environmental conditions (12-h light/dark cycles with lights on at 7:00 a.m., ambient temperature of 21 ± 1 °C, 45 ± 5% relative humidity) with ad libitum access to food and water, unless specifically noted. All experiments were carried out in accordance with the guidelines of the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n°113/13, and approved by ORBEA, the internal committee of the Faculty of Medicine, University of Porto (Portugal). Body weights were determined weekly before the experiments, on the day of surgery and once per week thereafter. To determine the average 24-h baseline food and water intake, the amount of food and water ingested was measured daily. At 10 weeks of age, rats were randomly assigned to one of three groups: control, nLOT-lesioned and sham-lesioned. nLOT- and sham-lesioned rats were allowed 10 days to recover prior to the start of behavioral testing.

Surgical procedures and stereotaxic injections

Rats were anesthetized by sequentially injecting, at intervals of 10 min, solutions of promethazine (10 mg/kg, s.c.; Laboratórios Vitória, Amadora, Portugal), followed by xylazine (2.6 mg/kg, i.m.; Sigma-Aldrich Company Ltd., Madrid, Spain), and finally, ketamine (50 mg/kg, i.m.; Merial Portuguesa, Rio de Mouro, Portugal), and placed on a stereotaxic apparatus with bregma and lambda in the same horizontal plane. nLOT lesions were made by bilateral infusion of 0.24 µL of quinolinic acid (Sigma-Aldrich). The acid was dissolved in 0.1 M of phosphate buffered saline (PBS) to a concentration of 180 nmol/µL (pH 7.1). The infusion of the toxin was done with a 1-µL Hamilton syringe (7001 N; Hamilton Bonaduz AG, Bonaduz, Switzerland). After a midline skin incision, holes were drilled bilaterally on the skull 1.2 mm posterior and

±3.2 mm laterally to the bregma (Paxinos and Watson 1998). The syringe was lowered into the brain until the depth of 9.2 mm from the bregma. The toxin was injected gradually (0.04 µL every 1.5 min) until the total amount was delivered. The needle was left in place for an additional 10 min, and then slowly withdrawn. The incisions on the skin were closed with surgical stitches and treated with local antiseptic. After surgery, rats were maintained in a warm place until recovery from anesthesia. Postoperative care consisted of subcutaneous injections of 0.9% physiological saline (2 mL) to prevent dehydration and weight loss. Sham lesions were similarly made by lowering the infusion needle at the same coordinates without infusing quinolinic acid.

Behavioral studies

All behavioral experiments were conducted during the standard light phase, starting at 2:00 p.m., except for sexual behavioral and aggression testing that was started 1 h after the beginning of the dark phase. For control (n = 30), sham-lesioned (n = 30) and nLOT-lesioned (n = 30) rats, tests were performed in the following order, with 1-day inter-test intervals: buried food test, olfactory habituation/cross-habituation test, open-field, elevated plus-maze, sucrose preference and fear conditioning. At the end of these tests, one third of the rats in each group (n = 10/group) were submitted either to (1) olfactory preference tests, (2) sexual behavior followed by aggression, or (3) Morris water maze followed by forced swim test (Fig. 1).

Buried food test

To assess the ability of the rats to smell volatile odors and their tendency to use olfactory cues for foraging, the buried food test (Yang and Crawley 2009) was performed. For odor familiarization, a highly palatable cookie was placed in the test chamber during two consecutive days before the test, and confirmed that it was consumed by the rat. The chamber consisted of a clean standard plastic cage (44 × 34 × 20 cm) with a 5 cm layer of new bedding. After 18 h of food deprivation, the rat was placed in the chamber for 10 min to acclimate. Then, the rat was removed from the cage and a cookie was buried in the bedding, approximately 2 cm beneath the surface, at a random location. The bedding surface was smoothed out and the rat was re-introduced into the cage. The time spent to locate the buried cookie was recorded. The maximum test time allowed was 900 s. Two hours later, a surface cookie test was performed. This test was set up in the same

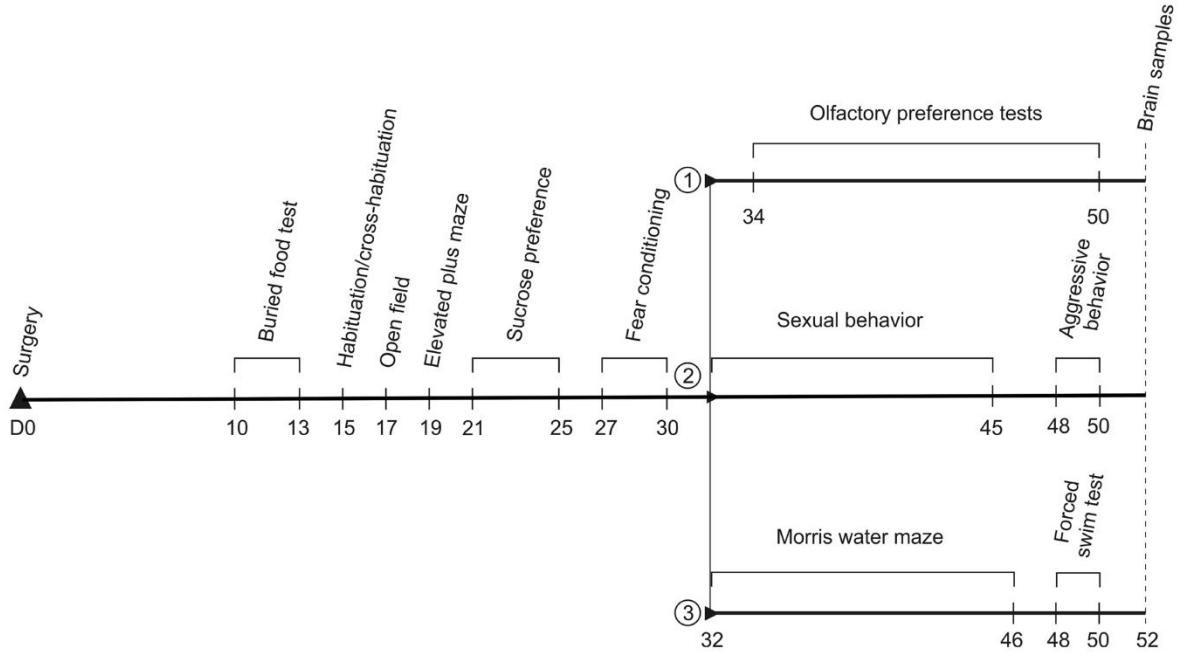


Fig. 1 Sequence and experimental timeline of the behavioral tests. The day of surgery was used as day 0 (D0) of the experiments. Control rats were age-matched. Tests were all done with 1-day inter-test intervals. Tests done until day 30 post-surgery included all rats (n = 30/group). Thereafter, one third of the rats in each group (n = 10/group) were submitted either to (*1*) olfactory preference tests, (*2*) sexual behavior followed by aggression, or (*3*) Morris water maze followed by forced swim test

way as the buried food test, but the cookie was placed on the surface of the bedding instead of being buried.

Olfactory habituation/cross-habituation test

To assess the ability to detect and discriminate between a familiar and a novel odor, the test was performed as previously described (Yang and Crawley 2009). Prior to testing, the rat was allowed to acclimate for 10 min to a clean cage (38 × 24 × 20 cm), in which a plastic tube applicator (10 × 0.5 cm), containing a rectangular filter paper inside (3 × 1 cm), was inserted through the water bottle hole. We used this kind of applicator to avoid direct contact with the odor stimulus. The test consisted of sequential presentations of water and four different odors in three consecutive trials of 2 min, with 1-min inter-trial intervals. The sequence used was water, two nonsocial odors (100 µL lemon extract, 1:10 dilution in distilled water, and 100 µL strawberry extract, 1:10 dilution in distilled water) and two different social male odors (100 µL of social odor solution). Each social odor was obtained by swabbing, with a cotton tip, the bedding of two different cages with male rats and diluting it in a glass vial with water and with 100 µL of urine collected from each of the males in those cages. The cumulative time spent sniffing the odorant in each presentation was quantified with a stopwatch within a 2-min time period.

Open-field test

To assess general exploratory locomotion and anxiety-like behaviors, we used an open-field apparatus that consisted of a white acrylic arena (100 × 100 × 40 cm). The rat was placed in a corner of the apparatus and tested during 5-min sessions. Distances travelled in the outer zone of the open-field, defined as 20 cm from any wall, and in its inner zone, defined as the 60 × 60 cm square in the center of the arena, were measured using a computerized video-tracking system (EthoVision XT 8.5, Noldus, The Netherlands). At the end of each session, the number of fecal boli deposited was counted and recorded, and the urine deposited was collected using a filter paper. The difference between the weight (in g) of the paper before and after collecting the urine was considered as a measure of the amount of urine deposited during the session. The floor of the apparatus was then thoroughly cleaned and dried.

Elevated plus-maze

To further evaluate general exploratory and anxiety-like behaviors, an elevated plus-maze apparatus consisting of a black acrylic cross with two opposite open and two opposite closed arms (50 × 12 cm) joined by a common central square (12 × 12 cm) was used. The closed arms were enclosed by 50-cm high walls. The test rat was placed on the central square facing one of the closed arms and allowed to explore the apparatus for 5 min. The behavior of the rat was recorded and analyzed using a computerized video-tracking system (EthoVision XT 8.5, Noldus). The percentages of time spent and the distances travelled by rats in the open arms, in the closed arms and in the central square were computed. At the end of each session, the number of fecal boli and the amount of urine were recorded. The apparatus was then thoroughly cleaned and dried.

Sucrose preference test

To assess possible anhedonia (decreased sensitivity to reward), generally associated to depression, rats were subjected to the sucrose preference test. During four consecutive days, rats were given the choice to drink from two bottles placed side-by-side, one containing water and the other a 2% of sucrose (Sigma-Aldrich) solution. One day before the test, rats were acclimated to the two-bottle configuration and to sucrose taste. The total amount of liquid consumed by rats was measured every day and fresh solutions were prepared. To avoid possible effects of bottle side bias, the position of the bottles was switched every 12 h. Sucrose preference was calculated as a percentage of the volume of sucrose solution intake relative to the total volume of fluid consumption and averaged over the 4-day testing period. The increase in the cumulative intake of water and sucrose solution during the 4-day test period was calculated by relation to the fluid intake measured under baseline conditions.

Fear conditioning

To assess the acquisition, consolidation and expression of fear conditioned responses, all rats were given a single session of fear conditioning, as previously described (Cardoso et al. 2009). The conditioning chamber (San Diego Instruments, USA) consisted of a clear Plexiglas box (26 × 26 × 18 cm) equipped with a metal grid floor connected to a stimulus generator (Hugo-Sachs Elektronik, Germany) and a buzzer. The grid floor was made of stainless steel bars (0.6 cm diameter) spaced 1.4 cm apart. An olfactory cue was added by placing 1% of acetic acid solution on the metal tray beneath the grid floor. On day 1, rats were allowed to explore the test chamber for 3 min. During the next 3-min period, they received 5 tone-foot-shock conditioning trials, with 30-s intervals. In each conditioning trial, rats were exposed to the conditioned stimulus (2.8 kHz, 80 dB tone lasting 10 s) co-terminated with the unconditioned stimulus (0.8 mA of a continuous 1-s footshock). Thirty seconds after the last trial, rats were

removed from the apparatus and the grid floor was cleaned with 1% acetic acid. Twenty-four hours later, half of the rats in each group were tested for contextual fear memory and the other half for cued fear memory. On the next day, rats already tested for contextual fear memory were tested for cue fear memory and vice versa. To test for contextual fear memory, rats were placed in the training chamber and were monitored for 6 min. To test for cued fear memory, rats were placed in a novel chamber, where they were left undisturbed for 3 min; then, during the next 3 min, they were exposed five times to the conditioned stimulus. In this test, the novel chamber was located in a novel behavioral room and was composed of black Plexiglas, except the top that was translucent and the floor that consisted of a piece of a black carpet and was scented with lemon instead of acetic acid. Training and all testing trials were recorded with a video camera for subsequent analysis. Freezing (defined as the absence of all movement other than that required for breathing and associated with a crouching posture) was scored if the rat remained inactive for at least 3 s. The percentage of accumulated time spent freezing was calculated.

Olfactory preference tests

Rats were allowed to habituate to the testing room and to the open-field arena during 10 min for 2 days prior to testing. Then, they were submitted, first, to a series of single odorant tests, and lastly, to the triple odorant test. Rats were exposed to one stimulus per day. They were naïve to each odor (as the odors tested were different from those used in the habituation/cross-habituation test) and were tested only once for each odor (at each concentration).

In the single odor test, the open-field arena contained Petri dishes in each corner, with only one Petri dish containing the odor stimuli. In the two habituation trials, the conditions were the same of the experimental test, except that no odor was used. Exposure to the odor was done by placing a 3.5-cm covered Petri dish containing a piece of filter paper (2 × 2 cm) impregnated with the odor stimuli in one corner of the open-field apparatus. As done by other authors (Dewan et al. 2013), the top of Petri dish was perforated to allow odorants to escape and to prevent direct nasal contact. One Petri dish containing a filter paper without any odor was placed in each of the other corners of the open-field arena. Because animals were tested in the same open-field arena to all odors, each of these odors was introduced in a pseudo-random corner of the arena to avoid any corner bias. Then, the test rat was placed in the center of the arena and allowed to freely explore the apparatus during 15 min. Sessions were video-recorded and later analyzed. The time spent by the rat actively investigating the corner where the odor was delivered was recorded and compared with the time rats spent investigating water. The odorants tested were 2-phenylethanol (2PE, 50% in water), 2-phenylethylamine (PEA, 50% in water), isopentylamine (IPA, 10% in water), 2,5-dihydro-2,4,5-trimethylthiazoline (TMT, 2% in water) and cat fur odor (CFO). With the goal of testing whether odor concentration would affect odor preference, we have also performed the test using concentrations that were twice higher for 2PE (100%), PEA (100%), IPA (20%) and five times larger for TMT (10%). Chemicals were all purchased from Sigma-Aldrich, except TMT, which was purchased from SRQBio (USA). Cat fur was collected from a domestic adult male cat.

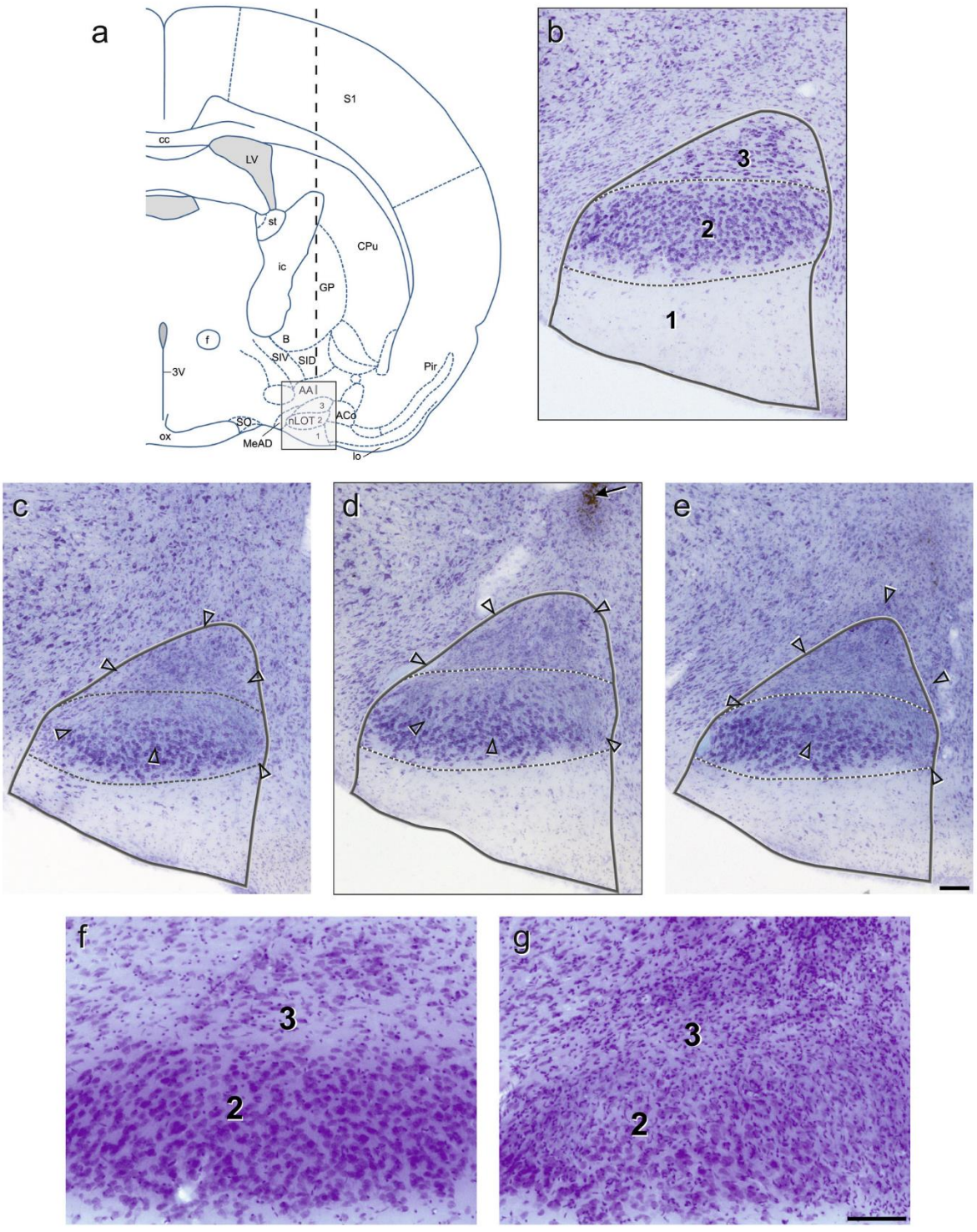
For the triple odor exposure, each rat was simultaneously presented with urine from group-housed testes-intact male rats, females in behavioral estrus and diestrus female rats. Urine was collected using metabolic cages. Urine from each group was pooled, aliquoted and stored at −80 °C until use. All the procedures were identical to the single odor test, except that in this test three odors and water were presented simultaneously, one in each corner. After placing the water and the three odor stimuli in the corners of the open-field cage, the test rat was placed in the center of the arena and allowed to freely explore for 15 min. Sessions were video-recorded and later analyzed. The time spent actively investigating each corner of the open-field was recorded.

Sexual behavior

Rats were observed in pre-copulatory behaviors and mounting in three assays with 4-days inter-trial intervals. On each trial, the test rat was placed into the cage and allowed to acclimate for 10 min. A sexually experienced receptive female rat was then introduced into the cage. Females were brought into behavioral estrus by subcutaneous injections of 10 μg of estradiol benzoate 52 h before the test, followed by 500 μg of progesterone 4–6 h before testing. Sexual behavior was recorded with a video camera during a 10-min period. The latency to the first anogenital exploration and to the first mounting/intromission was recorded from the time the female entered the cage. The cumulative duration of anogenital exploration, sniffing and rearing, and female pursuit was also recorded.

Aggressive behavior

Male aggression was assessed by the resident/intruder assay (Leypold et al. 2002). Rats in all groups were single-housed since the beginning of the study. The test rat was maintained in its home cage without changing of the bedding for the previous 4 days. A group-housed, sexually and aggressively inexperienced and unfamiliar adult rat, lighter than the test rat, was introduced into the home cage of the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◀**Fig. 2** **a** Schematic drawing of a coronal section of the rat brain through the nLOT (adapted from Paxinos and Watson 1998). The *thick dashed vertical line* indicates the trajectory of the needle. (**b–e**) Digital photomicrographs of Giemsa-stained coronal sections of the nLOT of a sham-lesioned rat (**b**) and a nLOT-lesioned rat (**c–e**). The *light-gray box* drawn in **a** delineates approximately the area where the photomicrographs shown in **b** and **d** were taken. The **c** and **e** sections are located 160 µm rostral and caudal, respectively, to the **d** section. In **b–e**, the nLOT is outlined by a *continuous line* and the borders between the adjacent layers are indicated by *dashed lines*. The *open arrowheads* in **c–e** demarcate the periphery of the lesion and the *arrow* in **d** indicates the injection track. (**f–g**) Photomicrographs of Nissl-stained coronal sections of the nLOT layers *2* and *3* of a sham-lesioned rat (**f**) and a nLOT-lesioned rat (**g**) taken at a higher magnification than those shown in **b–e** to demonstrate that, by comparison with section of the sham-lesioned rat (**f**), there are numerous reactive glial cells in the nLOT-lesioned rat (**g**). *3V* 3rd ventricle, *AA* anterior amygdaloid area, *ACo* anterior cortical amygdaloid nucleus, *B* basal nucleus (Meynert), *cc* corpus callosum, *CPu* caudate putamen (striatum), *f* fornix, *ic* internal capsule, *GP* globus pallidus, *lo* lateral olfactory tract, *LV* lateral ventricle, *MeAD* medial amygdaloid nucleus, anterodorsal part, *nLOT* nucleus of the lateral olfactory tract (layers *1*, *2* and *3*), *ox* optic chiasm, *Pir* piriform cortex, *S1* primary somatosensory cortex, *SID* substantia innominata, dorsal part, *SIV* substantia innominata, ventral part, *SO* supraoptic nucleus, *st* stria terminalis. *Scale bars* 100 µm

test rat. The tests were video-recorded over 15 min, and later analyzed to determine the duration of offensive behaviors (attack, offensive upright, lateral threat, keep down) and defensive behaviors (move away, submissive posture, defensive upright).

Morris water maze

To assess spatial learning and memory, rats were tested in a black circular pool (180 cm diameter; 50 cm deep) filled with water at room temperature (21 ± 1 °C) that was located in a corner of a room containing extra-maze cues. The pool was virtually divided into four equal-size quadrants. A black escape platform (10 cm in diameter) was placed in the center of one of the quadrants, 2 cm below the water surface. Swim paths were recorded by a computerized video-tracking system (EthoVision XT 8.5, Noldus). In the place learning task, rats were trained to find the submerged escape platform and to climb on it. For acquisition, rats were given two trials per day for 14 consecutive days, as follows. The test rat was placed in the water facing the pool wall at one of four starting points, which were used in a pseudo-random order so that each position would be used just once in each block of four trials. When rats did not find the escape platform within 60 s, the experimenter guided them to the platform where they were allowed to remain for 15 s. After the first daily trial, rats were placed in a clean cage for 30 s before the beginning of the next trial. The platform location was not changed during the acquisition period. The swim path length in each trial was measured. One day after the end of the acquisition period, rats were submitted to a single 60-s probe trial, in which the platform was removed from the pool. The number of times the rats swam through the zone where the platform had been located (platform crossings) and the time they spent swimming on the target and opposite quadrants were recorded. Starting one day later, all rats were tested during a 2-day period on the visible platform task to evaluate their sensorimotor abilities. In this task, rats were given 1 block of four trials per day separated by 30-s inter-trial intervals. The platform, painted in white, was exposed 3 cm above the water surface and its position was different in each trial. The distances swum to locate the platform were recorded and averaged across eight trials.

Forced swim test

To assess the depression state of animals, the forced swim test was done essentially as previously described (Porsolt et al. 1978). The apparatus used consisted of a transparent glass cylinder (25 cm diameter, 50 cm height) filled with tap water (23–25 °C) up to 30 cm from the bottom. On the first trial, the test rat was introduced into the apparatus and forced to swim during 15 min. On the day after, a second trial was performed during 5 min, and the behavior was recorded using a digital video camera. The total duration of immobility during the 5-min long testing period was recorded, and the percentage of accumulated time that rats were immobilized was calculated. Immobility was defined as the absence of movements beyond those required for keeping the head and nose above the water surface.

Brain tissue collection and histology

Following behavioral testing, rats were anesthetized with sevoflurane (SevoFlo, Abbott Laboratories Ltd, Maidenhead, UK) and blood samples were collected directly from the heart for hormone measurements. Then, rats were killed by transcardiac perfusion of 150 mL of 0.1 M phosphate buffer (PB), pH 7.6, for vascular rinse, followed by 250 mL of a fixative solution containing 4% paraformaldehyde in PB. The gonadal, perirenal and retroperitoneal fat depots were isolated, collected and weighed. The brains were removed from the skulls, immersed for 1 h in the same fixative, and maintained overnight in a solution of 10% sucrose in PB, at 4 °C. After removal of the frontal and occipital poles and separation of the right and left hemispheres, the remaining blocks of tissue were coronally sectioned at 40 µm on a vibratome throughout the rostrocaudal extent of the nLOT. Sections were alternately sampled, mounted on gelatin-coated slides, air-dried, either Giemsa- or Cresyl Violet (Nissl)-stained (Merck, Darmstadt, Germany), dehydrated and coverslipped with

Histomount (National Diagnostics, USA). Lesion placements were verified by microscopic examination, and drawn onto plates adapted from the atlas of Paxinos and Watson (1998). To assess the percent destruction of the nLOT, the volume of the nLOT cell layers and the volume of the lesions were estimated by point counting techniques (Gundersen and Jensen 1987; Madeira et al. 1997).

Imaging

Schematic presentations of the experimental design (Fig. 1), the coronal sections of the rat brain through the nLOT (Figs. 2a, 3) were made with Corel Draw X8 (version 18.1.0.661; Corel, Ottawa, CA, USA). The photomicrographs shown in Fig. 2b–g were captured by digital photography using a Axio Scope A1 microscope with an AxioCam MRc5 digital camera, the AxioVision software (version 4.8.1.0; Carl Zeiss, Göttingen, Germany) and the following objectives: 10x in Fig. 2b–e and 20× in Fig. 2f, g. Only minor adjustments of contrast and brightness were made using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA), without altering the appearance of the original materials. The plate photomontage and lettering were made with Corel Draw X8.

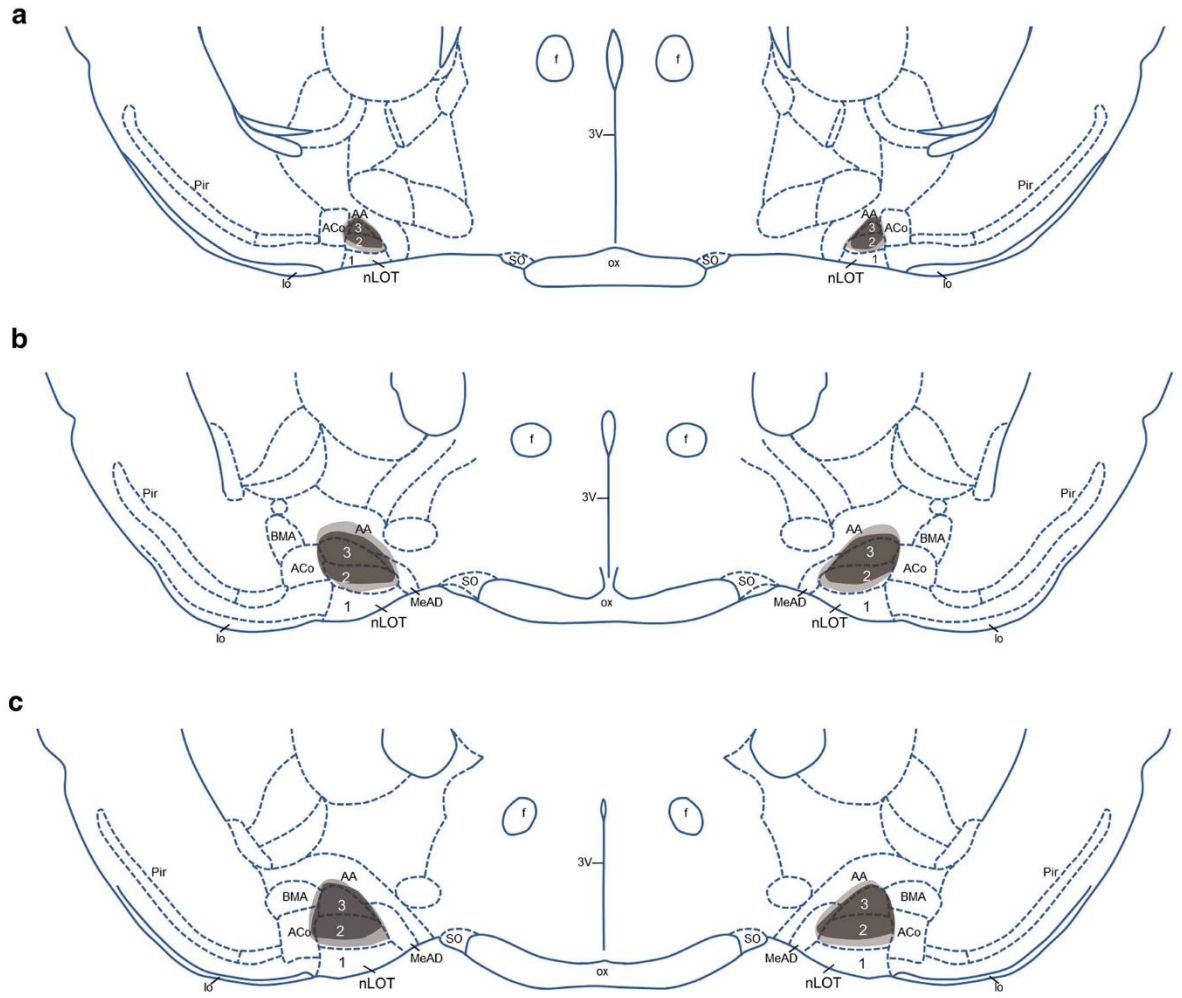

Fig. 3 Rostrocaudal sequence **a–c** of schematic drawings of coronal sections of the rat brain through the nLOT (adapted from Paxinos and Watson 1998). Shaded areas represent the maximal (*light gray*) and the minimal (*dark gray*) extent of the nLOT lesions that were observed in the rats included in the study. *3V* 3rd ventricle, *AA* anterior amygdaloid area, *ACo* anterior cortical amygdaloid nucleus, *BMA* basomedial amygdaloid nucleus, anterior part, *f* fornix, *lo* lateral olfactory tract, *MeAD* medial amygdaloid nucleus, anterodorsal part, *nLOT* nucleus of the lateral olfactory tract (layers *1*, *2* and *3*), *ox* optic chiasm, *Pir* piriform cortex, *SO* supraoptic nucleus

Hormone measurements

The blood samples collected at the time of killing were centrifuged. The serum was collected and immediately frozen at −80 °C until assayed. Leptin was measured using a commercial ELISA kit (Rat Leptin ELISA Kit; ab100773). Sensitivity of the test was 30 pg/mL and the intra and inter-assay coefficient of variation was 5.75 and 8.98%, respectively. Testosterone was determined by enzyme-linked fluorescent assay using VIDAS Testosterone Kit and mini VIDAS analyzer (bioMerieux S.A., Marcy L'Etoile, France). According to the manufacturer, the assay has a measurement range of 0.1–13 ng/mL. The analyzer was cleaned, calibrated, and operated in accordance with the manufacturer's instructions. For testosterone, all samples were tested in singlicate, analyzed in the same assay and the intra-assay coefficient of variation was 7.44%.

Statistical analyses

All figures represent means with the error bars representing SEM. Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA). Repeated measures analysis of variance (ANOVA) was used to analyze data on body weights (independent variables: post-surgery time and treatment; dependent variable: body weight), relative body weight change (independent variables: post-surgery time and treatment; dependent variable: body weight change relative to pre-lesion body weight), relative food intake (independent variables: post-surgery time and treatment; dependent variable: relative food intake) and fear conditioning (independent variables: context, tone and treatment; dependent variable: time spent in freezing). Two-way ANOVA was used for analysis of data obtained in the buried food test (independent variables: cookie location, treatment; dependent variable: time spent to find the cookie), open-field (independent variables: zone and treatment; dependent variables: distance travelled, number of fecal boli and grams of urine) and elevated plus-maze (independent variables: zone and treatment; dependent variables: distance travelled, time spent, number of fecal boli and grams of urine). One-way ANOVA was used to statistically analyze body fat mass and hormone levels (independent variable: treatment; dependent variables: fat mass, leptin levels, testosterone levels), and data from the sucrose preference test (independent variable: treatment; dependent variables: sucrose preference and fluid intake increase), forced swim test (independent variable: treatment; dependent variable: time spent in immobility), sexual behavior (independent variable: treatment; dependent variables: latency to anogenital exploration, latency to first mount, cumulative time spent in anogenital exploration, time spent sniffing and rearing and time spent in female pursuit), aggressive behaviors (independent variable: treatment; dependent variable: time spent in offensive behaviors) and defensive behaviors (independent variable: treatment; dependent variable: time spent in defensive behaviors). Data on olfactory habituation/cross-habituation was analyzed using repeated measures ANOVA (independent variables: water, lemon, strawberry, social 1, social 2, treatment; dependent variable: time spent sniffing the odor), whereas the cumulative time spent by rats sniffing each odor over the three trials was analyzed using a one-way ANOVA. Data obtained in the single odor test applied to examine olfactory preference, compared to water, was analyzed using two-way ANOVA (independent variables: 2PE, PEA, IPA, CFO, TMT, treatment; dependent variable: time spent in odor corner), whereas the triple odor test was analyzed separately for each treatment group by one-way ANOVA (independent variables: water, non-receptive female, receptive female and male odors; dependent variable: time spent in the odor corners). Data obtained in the Morris water maze was analyzed using repeated measures ANOVA (independent variables: platform location, treatment; dependent variable: distance travelled to find the platform) as well as a two-way ANOVA (independent variables: platform and treatment; dependent variable: time spent in the target and opposite quadrants). Whenever appropriate, ANOVAs were followed by Tukey highest significant difference (HSD) post-hoc comparisons. Differences were considered to be statistically significant when $p < 0.05$.

Results

Lesion analysis

The injection tracks, the placement of each injection site, and the location and size of the lesions were subjected to rigorous histological examination through microscopic analysis of Giemsa- and Nissl-stained sections (Fig. 2) before analysis of the behavioral data. The needle track passed through the cerebral cortex, corpus callosum, caudate-putamen, internal capsule, globus pallidus, basal nucleus (Meynert), substantia innominata and the anterior amygdaloid area (Fig. 2a). Minor mechanical damage from the needle insertion was evident in both sham- and nLOT-lesioned rats. None of the sham-lesioned rats presented damage of the nLOT or neighboring structures (Fig. 2b, f). Lesions were classified as suitable if there was significant bilateral damage encompassing an area greater than 50% of the nLOT cell layers, and no noteworthy damage of the neighboring structures, namely the anterior amygdaloid area, the basomedial, anterior cortical and medial amygdaloid nuclei, the lateral olfactory tract, the piriform cortex or the hypothalamus. Rats that did

not fulfill these criteria, either unilaterally or bilaterally, were not included in the study, i.e., rats in which lesions could not be identified accurately ($n = 3$) and rats that only had unilateral lesions ($n = 2$) or presented significant bilateral damage of neighboring structures ($n = 3$). In all nLOT-lesioned rats included in the study there was massive neuronal loss and gliosis in layer 3 with variable, but frequently prominent damage of layer 2 (Fig. 2c–e, g). Taking into account the size of the lesions in coronal sections and their extension along the rostrocaudal length of the nLOT, the lesions of the rats included in the study encompassed, on average, 65% of the nLOT cell layers. Representative examples of nLOT lesions are shown, in a rostrocaudal sequence, in Fig. 2c–e. The maximal and the minimal extent of the lesions are depicted, also in a rostrocaudal sequence, in Fig. 3.

Body weight and composition

Body weights were significantly influenced by treatment ($F_{2,87} = 7.7$, $p < 0.001$) and post-surgery time ($F_{1,87} = 907.0$, $p < 0.001$); a significant treatment × post-surgery time was also found ($F_{2,87} = 17.0$, $p < 0.001$). At the beginning of the experiments, rats in all groups had similar body weights (Table 1). During the experiments, the body weight variations relative to pre-lesion body weights (Fig. 4a) were significantly influenced by treatment ($F_{2,87} = 62.5$, $p < 0.001$) and post-surgery time ($F_{7,609} = 742.8$, $p < 0.001$); a significant treatment × post-surgery time interaction was also found ($F_{14,609} = 29.1$, $p < 0.001$). nLOT- and sham-lesioned rats initially showed a significant weight loss, which reached, on average, 8 and 5%, respectively, of the preoperative body weight at 1 week after surgery, followed by a progressive increase until the end of the experiments. During weeks 2–5 post-surgery, the

Table 1 Summary of the effects of nLOT and sham lesions on body weights, fat mass, leptin and testosterone levels

	Control	Sham	nLOT lesion
Body weight (g)			
Day of surgery	375 (4)	361 (4)	361 (6)
End of experiment	435 (4)*	401 (5)*	404 (8)*
Fat mass (% bw)	4.1 (0.1)	2.9 (0.1)#	3.2 (0.1)#
Leptin (ng/mL)	5.3 (0.09)	4.3 (0.09)#	4.4 (0.06)#
Testosterone (ng/mL)	2.1 (0.28)	1.9 (0.26)	2.5 (0.23)

Values are expressed as mean (SEM)

* $p < 0.001$ compared with initial body weight of the respective group

# $p < 0.001$ compared with control rats

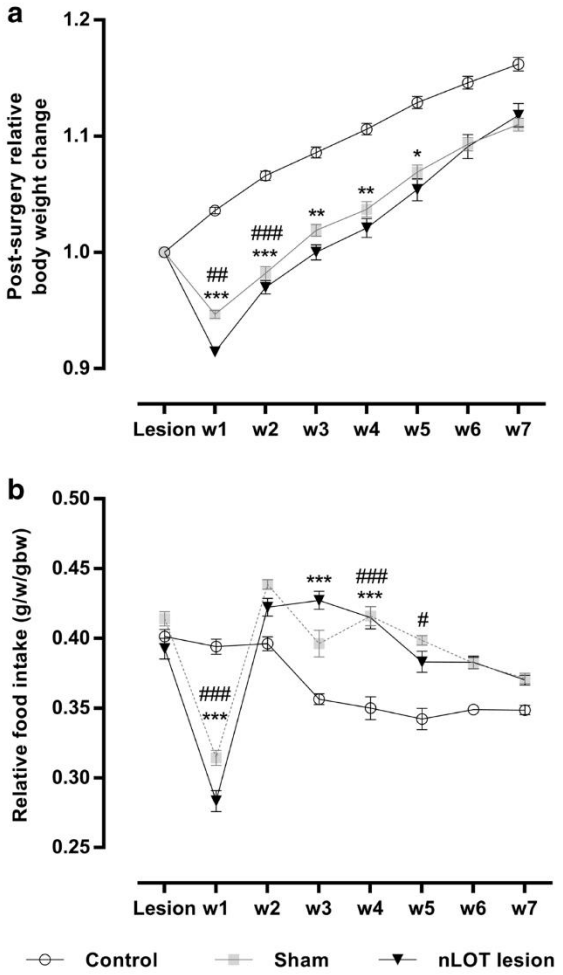

Fig. 4 Relative body weight and food intake variations following the day of surgery for inducing nLOT or sham lesions, presented as mean ± SEM. **a** Body weight change, expressed as a percent of the average weekly gain or loss from pre-surgery weight. One week after surgery, nLOT- and sham-lesioned rats showed significant weight loss compared to controls, followed by a progressive increase until the end of the experiments. During week-2 post-surgery, the relative body weight gain was significantly higher in nLOT- and sham-lesioned rats than in controls. From weeks 3 to 5, only in nLOT-lesioned rats the relative weight gain was significantly higher than in controls. Thereafter, the relative body weight variations were similar in all groups. **b** Relative food intake, expressed as grams of food intake per week, per gram body weight. The relative food intake was smaller in nLOT- and sham-lesioned rats compared to controls during week-1 post-surgery. Thereafter, there was a tendency for the relative food intake to be higher in nLOT- and sham-lesioned rats than in controls. However, the differences reached statistical significant levels only at weeks 3 and 4 for nLOT-lesioned rats, and weeks 4 and 5 for sham-lesioned rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between nLOT-lesioned and controls; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ between sham-lesioned and control rats

relative body weight gain was significantly higher in nLOT-lesioned rats than in controls (Fig. 4a), whereas for sham-lesioned rats the relative body weight was significantly higher only in week 2. Thereafter and until the day of killing, the relative body weight gain did not significantly differ among nLOT-lesioned, sham-lesioned and control rats. Consequently, at the end of the experiments, nLOT- and sham-lesioned rats were significantly less heavy than control rats (Table 1).

Fat mass, expressed as a percentage of body weight, was significantly influenced by treatment ($F_{2,87}$ = 25.3, $p < 0.001$). It did not differ between nLOT- and sham-lesioned rats, and was, in both groups, significantly smaller than in controls (Table 1).

Food intake

The relative food intake (g/week/g body weight) was influenced by treatment ($F_{2,87}$ = 8.9, $p < 0.001$) and post-surgery time ($F_{7,609}$ = 93.2, $p < 0.001$); a significant treatment × post-surgery time interaction ($F_{14,609}$ = 44.0, $p < 0.001$) was also found. Baseline pre-surgery relative food intake was similar in all groups at the beginning of the experiments (Fig. 4b). During week-1 post-surgery, the relative food intake became significantly smaller in nLOT- and sham-lesioned rats than in controls. Thereafter, there was a tendency for the relative food intake to be higher in nLOT- and sham-lesioned rats than in controls. However, the differences reached statistical significant levels only at weeks 3 and 4 for nLOT-lesioned rats, and weeks 4 and 5 for sham-lesioned rats.

Hormone levels

These data are shown in Table 1. Serum leptin levels were significantly influenced by treatment ($F_{2,87}$ = 51.5, $p < 0.001$). They were similar in nLOT- and sham-lesioned rats, but were in both groups significantly lower

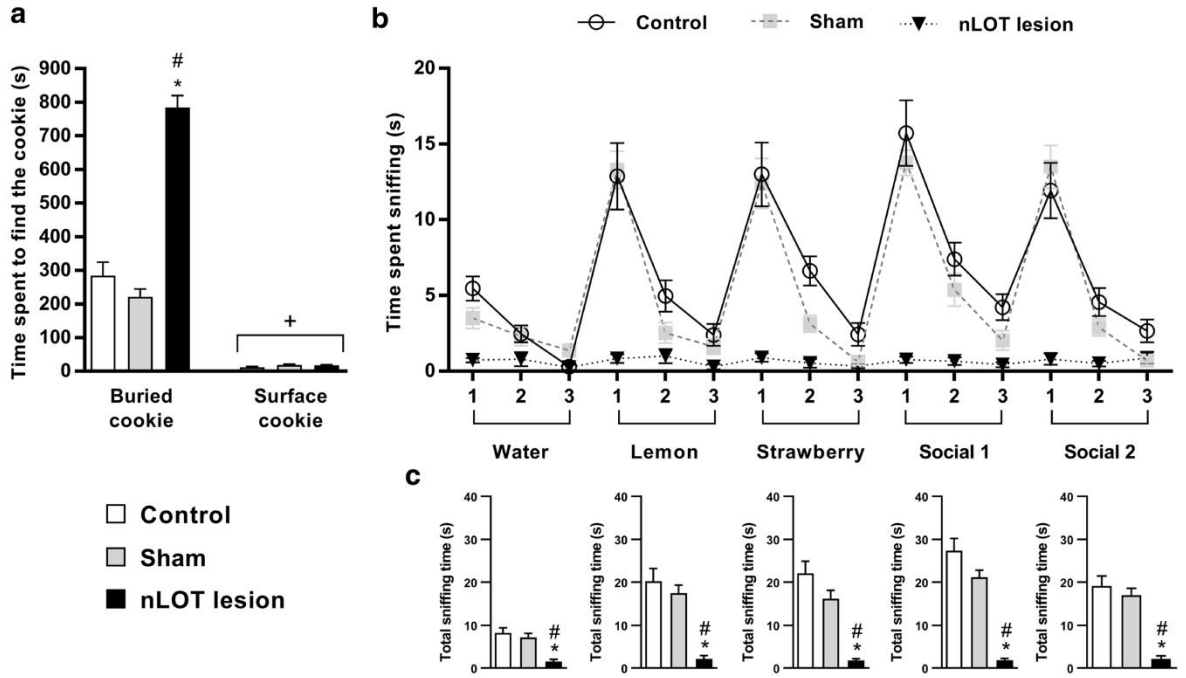

Fig. 5 nLOT-lesioned rats have severe olfactory deficits. **a** Buried food test. Histograms show the mean + SEM time spent to find the hidden and the surface cookies. The latency to find the hidden cookie was significantly longer in nLOT-lesioned than in sham-lesioned and control rats. No differences were found between groups to find the visible cookie. **b, c** Olfactory habituation/cross-habituation test. The graph **b** shows mean ± SEM olfactory investigation times across three consecutive 2-min trials separated with 1-min inter-trial of water, two nonsocial and two social odors. Control and sham-lesioned rats, but not nLOT-lesioned rats, show significantly shorter investigation times across three presentations of the same odor (habituation). In addition, control and sham-lesioned rats, but not nLOT-lesioned rats, significantly increased the investigation time after presentation of a new odor (cross-habituation). No significant differences were found between the investigation times of control and sham-lesioned rats. The values of the post-hoc tests are shown in the "Results" section. The histogram **c** shows that the mean + SEM cumulative time that nLOT-lesioned rats spent investigating each odor was significantly inferior to that spent by sham-lesioned and control rats. $^{*}p < 0.001$ compared to controls; $^{\#}p < 0.001$ compared to sham-lesioned rats; $^{+}p < 0.001$ compared to the respective group in the buried food test

than in controls. Conversely, serum testosterone levels did not differ among all groups ($F_{2,87}$ = 1.3, n.s.).

nLOT-lesioned rats have severe olfactory deficits

As shown in Fig. 5a, there was a significant influence of treatment ($F_{2,174}$ = 84.3, $p < 0.001$) and cookie location ($F_{1,174}$ = 453.9, $p < 0.001$) on the latency to find the cookie; a significant treatment × cookie location interaction ($F_{2,174}$ = 83.2, $p < 0.001$) was also found. The average latency to find the hidden cookie was significantly longer in nLOT-lesioned than in sham-lesioned and control rats. No differences were found between sham-lesioned and control rats. Conversely, there were no differences among groups in the time spent to find the visible cookie.

In the olfactory habituation/cross-habituation test (Fig. 5b), repeated measures ANOVA showed that rats in all groups responded to the sequential presentation of the five odors ($F_{14,1218}$ = 48.3, $p < 0.001$), and that there was a significant influence of treatment ($F_{2,87}$ = 51.0, $p < 0.001$) in the amount of olfactory investigation of the different odors. The analysis of individual odors showed that there was a significant effect of treatment in the response to water ($F_{2,87}$ = 15.1, $p < 0.001$), lemon ($F_{2,87}$ = 21.4, $p < 0.001$), strawberry ($F_{2,87}$ = 26.4, $p < 0.001$), social 1 ($F_{2,87}$ = 49.7, $p < 0.001$) and social 2 ($F_{2,87}$ = 30.7, $p < 0.001$) odors. Post-hoc analysis of these data revealed that nLOT-lesioned rats spent significantly less time sniffing water, nonsocial and social odors than control and sham-lesioned rats ($p < 0.001$, for all odors) and that there were no differences between control and sham-lesioned rats. As expected, when the cumulative time that animals spent sniffing each odor over the three trials was analyzed, a significant effect of treatment was also found: water ($F_{2,87}$ = 15.1, $p < 0.001$), lemon ($F_{2,87}$ = 21.4, $p < 0.001$), strawberry ($F_{2,87}$ = 26.4, $p < 0.001$), social 1 ($F_{2,87}$ = 49.7, $p < 0.001$) and social 2 ($F_{2,87}$ = 30.7, $p < 0.001$) odors (Fig. 5c).

When each experimental group was individually analyzed, a significant habituation to each odor was observed in control rats, as shown by the progressive decline in the time spent sniffing water ($F_{2,58}$ = 25.6, $p < 0.001$), lemon ($F_{2,58}$ = 17.5, $p < 0.001$), strawberry ($F_{2,58}$ = 18.0, $p < 0.001$), social 1 ($F_{2,58}$ = 17.9, $p < 0.001$) and social 2 ($F_{2,58}$ = 16.0, $p < 0.001$) odors. The same happened with sham-lesioned rats for water ($F_{2,58}$ = 3.5, $p < 0.05$), lemon ($F_{2,58}$ = 82.4, $p < 0.001$), strawberry ($F_{2,58}$ = 43.3, $p < 0.001$), social 1 ($F_{2,58}$ = 52.3, $p < 0.001$) and social 2 ($F_{2,58}$ = 63.2, $p < 0.001$) odors. Conversely, in nLOT-lesioned rats no habituation was found for any of the odors tested: water ($F_{2,58}$ = 0.8, n.s.), lemon ($F_{2,58}$ = 1.9, n.s.), strawberry ($F_{2,58}$ = 2.2, n.s.), social 1 ($F_{2,58}$ = 0.7, n.s.) and social 2 ($F_{2,58}$ = 0.5, n.s.) odors.

When evaluating cross-habituation, we found that there was a significant effect of treatment when a new odor was presented for the first time: water to lemon ($F_{2,87}$ = 25.4, $p < 0.001$), lemon to strawberry ($F_{2,87}$ = 20.6, $p < 0.001$), strawberry to social 1 ($F_{2,87}$ = 30.2, $p < 0.001$) and social 1 to social 2 ($F_{2,87}$ = 35.4, $p < 0.001$) odors. Post-hoc analysis showed that nLOT-lesioned rats responded significantly less than control and sham-lesioned rats when lemon ($p < 0.001$), strawberry ($p < 0.001$), social 1 ($p < 0.001$) and social 2 ($p < 0.001$) odors were presented for the first time. No significant differences were found between control and sham-lesioned rats. When each experimental group was individually analyzed, control and sham-lesioned rats showed a significant cross-habituation when presented for the first time to a new odor: water to lemon ($F_{1,29}$ = 33.2, $p < 0.001$ and $F_{1,29}$ = 89.0, $p < 0.001$, respectively), lemon to strawberry ($F_{1,29}$ = 27.7, $p < 0.001$ and $F_{1,29}$ = 39.7, $p < 0.001$, respectively), strawberry to social 1 ($F_{1,29}$ = 52.7, $p < 0.001$ and $F_{1,29}$ = 246.2, $p < 0.001$, respectively), and social 1 to social 2 ($F_{1,29}$ = 14.4, $p < 0.001$ and $F_{1,29}$ = 45.2, $p < 0.001$, respectively). In contrast to these groups, nLOT-lesioned rats could not discriminate between any of the odors tested: water and lemon ($F_{1,29}$ = 3.1, n.s.), lemon and strawberry ($F_{1,29}$ = 3.1, n.s.), strawberry and social odor 1 ($F_{1,29}$ = 3.2, n.s.), and social 1 and social 2 ($F_{1,29}$ = 0.7, n.s.) odors.

nLOT-lesioned rats have normal locomotor activity

Locomotor activity was assessed in the open-field and elevated plus-maze (Fig. 6). The distances, expressed in cm (SEM), travelled in the open-field were 3037 (115) for control rats, 3308 (96) for sham-lesioned rats and 3025 (131) for nLOT-lesioned rats. In the elevated plus-maze the total distances were 1652 (52), 1481 (70) and 1534 (61), respectively. There was no significant influence of treatment in the total distances travelled in the open-field ($F_{2,174}$ = 2.1, n.s.) as well as in the elevated plus-maze ($F_{2,261}$ = 2.2, n.s.), indicating no differences among groups in locomotor and exploratory activities.

nLOT-lesioned rats do not exhibit anxiety-like behaviors

We measured anxiety-like behavior in the open field and in the elevated plus-maze. There was no significant influence of treatment ($F_{2,174}$ = 2.1, n.s.), but there was a significant main effect of zone ($F_{1,174}$ = 2173.6, $p < 0.001$) on the distances travelled in the outer and in the inner zones of the open-field; no treatment × zone interaction was found ($F_{2,174}$ = 1.7, n.s.). Similar to control and sham-lesioned rats, nLOT-lesioned rats travelled longer distances in the

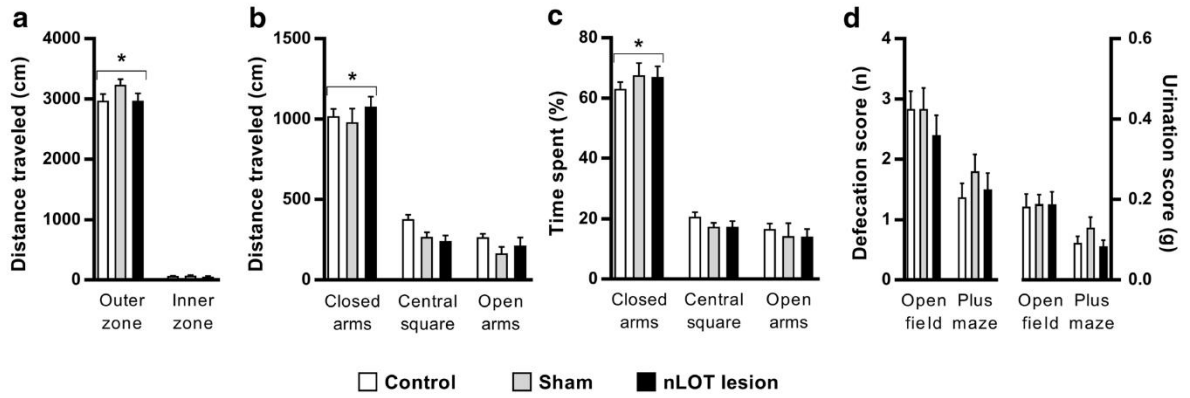

Fig. 6 nLOT-lesioned rats have normal locomotor activity and do not exhibit anxiety-like behaviors. **a** Open-field test. The histogram shows the mean + SEM distances travelled in outer and inner zones of the open-field. There were no significant differences between groups in the distances travelled in each zone, and similar to control and sham-lesioned rats, nLOT-lesioned rats travelled significant longer distances in the outer zone than in the inner zone. **b, c** Elevated plus-maze test. Graphic representation of the mean + SEM distances travelled (**b**) and time spent (**c**) in closed and open arms, and in central square of the elevated plus-maze. There were no significant differences between controls, sham-lesioned and nLOT-lesioned rats in the distances travelled and in the time spent in the closed and open arms, and in central square of the maze. In addition, rats of all groups travelled significantly longer distances and spent significantly more time in the closed arms than in the open arms and central square of the maze. **d** The histogram shows the mean + SEM number of fecal boli and amount of urine deposited by rats during the open-field and elevated plus-maze tests. No significant differences between the groups were found. $*p < 0.001$ compared with the inner zone in the open-field test and with the open arms and central square in the elevated plus-maze test

outer zone than in the inner zone of the open-field (Fig. 6a). The distances travelled and the time spent in the different zones of the elevated plus-maze were also significantly influenced by zone ($F_{2,261} = 262.7$, $p < 0.001$ and $F_{2,261} = 324.9$, $p < 0.001$, respectively), but not by treatment ($F_{2,261} = 2.2$, n.s. and $F_{2,261} = 0.04$, n.s., respectively); no treatment × zone interactions were found ($F_{4,261} = 1.1$, n.s. and $F_{4,261} = 0.8$, n.s., respectively). Rats of all groups travelled significantly longer distances (Fig. 6b) and spent significantly more time (Fig. 6c) in the closed arms than in the central square and opens arms of the elevated plus-maze, where rats of all groups spent approximately the same time and travelled approximately the same distances. No differences between groups were found in the open-field and in the elevated plus-maze, which indicates that nLOT lesions do not affect the state of anxiety.

There was also no significant effect of treatment on the defecation and urination scores (Fig. 6d) in the open-field ($F_{2,87} = 0.6$, n.s. and $F_{2,87} = 0.01$, n.s., respectively) as well as in the elevated plus-maze ($F_{2,87} = 0.7$, n.s. and $F_{2,87} = 1.5$, n.s., respectively).

nLOT-lesioned rats do not have depressive-like behaviors

No significant effect of treatment on sucrose preference was found among all groups ($F_{2,87} = 1.5$, n.s.; Fig. 7a), which indicates that nLOT lesions do not increase anhedonia and depressive-like responses. As shown in Fig. 7b, there were no differences among groups in the increase in fluid intake that occurred during the 4-day sucrose preference test relative to the baseline fluid intake of each group ($F_{2,87} = 0.1$, n.s.).

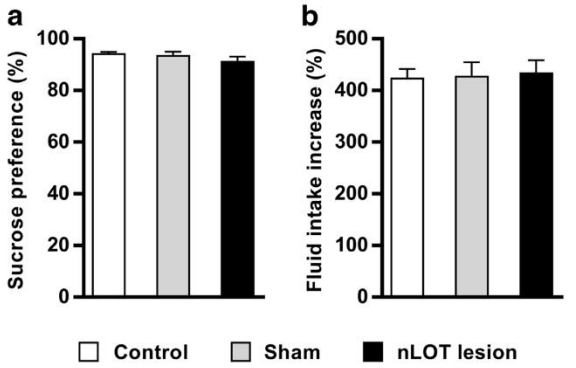

Fig. 7 nLOT-lesioned rats do not have depressive-like behaviors. **a, b** Sucrose preference test. The histogram in **a** shows the mean + SEM percentage of sucrose solution ingestion relative to the total amount of liquid consumption by each group of rats averaged over the 4 days of the test. No significant differences were found in sucrose preference between groups, indicating that nLOT lesions do not increase anhedonia or induce depressive-like behaviors. The histogram in **b** shows the mean + SEM percent increase in the total amount of fluid consumption (water plus sucrose solution) during the test relative to baseline water consumption. No differences among all groups were found

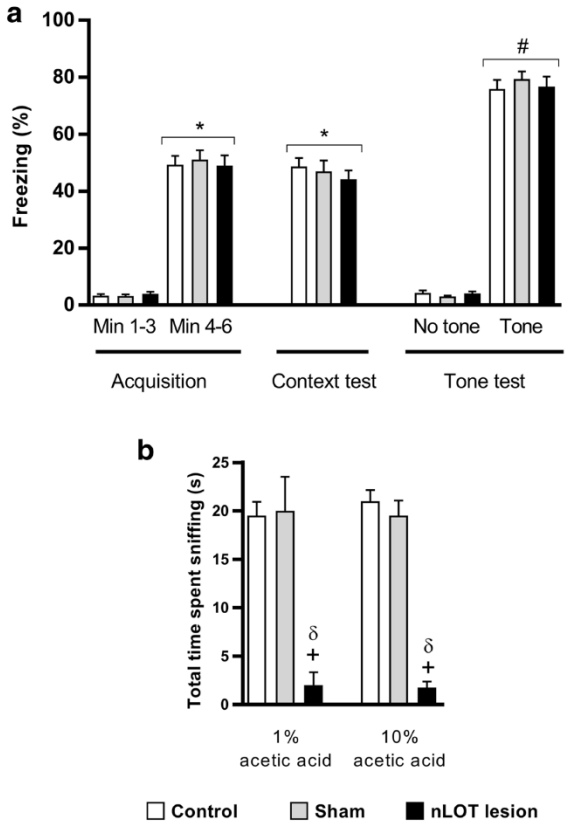

Fig. 8 nLOT-lesioned rats have similar levels of contextual and cued fear conditioned learning and memory. **a** nLOT- and sham-lesioned rats did not show any difference compared to control rats in the percentage of freezing time for each of the 3-min periods of the acquisition session, during the context retention test, and during each of the 3-min periods of the tone retention test, which was performed in a novel context. No tone or footshock was delivered during the first 3-min periods of the acquisition session and of the tone retention test. The histogram **b** shows that the mean cumulative time that nLOT-lesioned rats spent investigating 1% and 10% acetic acid was significantly inferior to that spent by sham-lesioned and control rats. Data are presented as the mean + SEM. $^{*}p < 0.001$ compared to min 1–3 of the acquisition test of the respective group; $^{\#}p < 0.001$ compared to the no tone period of the respective group; $^{+}p < 0.001$ compared to controls, $^{\delta}p < 0.001$ compared to sham-lesioned rats

nLOT-lesioned rats have unimpaired fear acquisition and memory

Data of the fear conditioning test (Fig. 8a) showed that conditioning increased the total time of freezing in all groups ($F_{1,87}$ = 588.6, $p < 0.001$). However, no effect of treatment ($F_{2,87}$ = 0.1, n.s.) and no treatment × conditioning interaction ($F_{2,87}$ = 0.2, n.s.) was found, which indicates a similar acquisition of fear during training in all groups. Measurement of conditioned fear 24 h post-training upon re-exposure to context revealed that all groups increased the total time of freezing ($F_{1,87}$ = 476.6, $p < 0.001$) and that there was no effect of treatment ($F_{2,87}$ = 0.4, n.s.) in contextual fear memory, which indicates that all groups have similar levels of contextual memory. When the rats were introduced into the novel context, there was no increase in the total time of freezing in all groups ($F_{1,87}$ = 0.3, n.s.). However, when rats were exposed to the conditioned stimulus (tone) there was a significant increase in total freezing time ($F_{1,87}$ = 1500.2, $p < 0.001$), and this effect was not dependent on treatment ($F_{2,87}$ = 0.2, n.s.) or on treatment × stimulus interaction ($F_{2,87}$ = 0.6, n.s.), showing that rats in all groups have similar levels of cued fear memory. To examine the relevance of the acetic acid odor for the normal response of nLOT-lesioned rats in the contextual fear conditioning, we analyzed, using a habituation/cross-habituation test, the ability of nLOT-lesioned rats to smell acetic acid in two different concentrations (1 and 10%). Our results show that there was a significant influence of treatment ($F_{2,18}$ = 63.3, $p < 0.001$), but not of acetic acid concentration ($F_{1,18}$ = 0.03, n.s.), in the amount of olfactory investigation (Fig. 8b).

nLOT-lesioned rats do not show innate attractive or avoidance behaviors

As shown in Fig. 9a, treatment significantly influenced the percentage of time spent in the odor corner of the open-field apparatus, compared to water, when rats were exposed to 2PE ($F_{2,54}$ = 3.3, $p < 0.05$), PEA ($F_{2,54}$ = 7.4, $p < 0.01$), IPA ($F_{2,54}$ = 4.8, $p < 0.05$) and CFO ($F_{2,54}$ = 10.4, $p < 0.001$), but not to TMT ($F_{2,54}$ = 2.1, n.s.). Similarly, odors also significantly influenced the percentage of time spent in the odor corner, compared to water, when rats were exposed to 2PE ($F_{1,54}$ = 12.1, $p < 0.001$), PEA ($F_{1,54}$ = 9.4, $p < 0.01$), IPA ($F_{1,54}$ = 13.4, $p < 0.001$), TMT ($F_{1,54}$ = 4.9, $p < 0.05$) and CFO ($F_{1,54}$ = 44.1, $p < 0.001$). Moreover, a significant treatment × odor interaction was also found for 2PE ($F_{2,54}$ = 4.7, $p < 0.05$), PEA ($F_{2,54}$ = 5.1, $p < 0.01$), IPA ($F_{2,54}$ = 3.7, $p < 0.05$) and CFO ($F_{2,54}$ = 8.4, $p < 0.001$), but not for TMT ($F_{2,54}$ = 1.1, n.s.). Because the behavioral response to TMT is concentration-dependent (Saraiva et al. 2016), we repeated the test with a concentration that was five times higher. The results obtained were similar to those elicited by the concentration of 80 mM (data not shown). No concentration-related effects were also detected for 2PE, PEA and IPA (data not shown). Control and sham-lesioned rats spent significantly more time in the corner containing 2PE and significantly less time in the corner containing PEA, IPA or CFO than in the corner containing water, which indicates that they displayed innate attraction to 2PE and innate aversion to

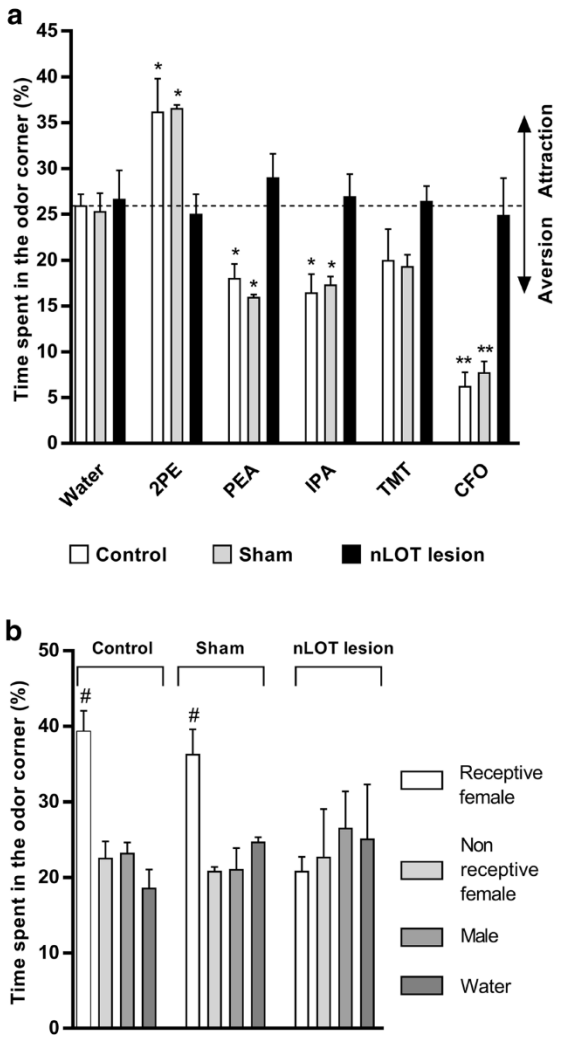

Fig. 9 nLOT-lesioned rats do not show innate odor attractive or aversive behaviors. The histogram in **a** shows the mean + SEM percentage of the cumulative time that rats spent investigating the corner of the open-field arena where the several odors were individually presented over the duration of the test. The percentage of time spent investigating water (*dashed line*) was used as the criterion to define the threshold between attraction and aversion. Control and sham-lesioned rats spent significantly more time sniffing the attractive odor 2-phenylethanol (2PE) and significantly less time sniffing the aversive odors 2-phenylethylamine (PEA), isopentylamine (IPA) and cat fur odor (CFO) than water. Conversely, nLOT-lesioned rats spent a similar time investigating water and all the other odors presented. **b** Graphic representation of the mean + SEM percentage of the cumulative time that rats spent investigating the corners of the open-field arena where the Petri dishes containing the odors of receptive females, non-receptive females, males and water (one in each corner) over the duration of the test. Control and sham-lesioned rats spent significantly more time sniffing the receptive female odor than the remaining odors. Conversely, nLOT-lesioned rats did spent a similar time investing all odors presented. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to water of the respective group; $^{\#}p < 0.001$ compared to other odors and water

PEA, IPA and CFO. Conversely, in nLOT-lesioned rats, 2PE, PEA, IPA, TMT and CFO did not elicit attractive or avoidance behaviors.

In the triple odor test, we found that the time spent by rats actively investigating the different corners of the open-field arena (Fig. 9b) was significantly influenced by odors in the control ($F_{3,36} = 17.5$, $p < 0.001$) and sham-lesioned ($F_{3,36} = 11.3$, $p < 0.001$) groups, but not in the nLOT-lesioned group ($F_{3,36} = 0.2$, n.s.). Control and sham-lesioned rats showed preference to investigate proestrus female odors over diestrus female or male odors and water, whereas nLOT-lesioned investigated all odors and water for a similar amount of time.

nLOT-lesioned rats have impaired sexual behavior

We found a significant influence of treatment in the latency to anogenital exploration ($F_{2,27} = 22.4$, $p < 0.001$) and to the first mount ($F_{2,27} = 651.6$, $p < 0.001$). A similar effect was also found in the percentage of cumulative time spent in anogenital exploration ($F_{2,27} = 34.5$, $p < 0.001$), sniffing and rearing ($F_{2,27} = 37.9$, $p < 0.001$) and female pursuit ($F_{2,27} = 80.5$, $p < 0.001$). The latency to anogenital exploration did not significantly differ between nLOT- and sham-lesioned rats and was, in both groups, significantly longer than in controls (Fig. 10a). The percent time spent by nLOT-lesioned rats in anogenital exploration (Fig. 10b) and in female pursuit (Fig. 10c) was significantly smaller than in sham-lesioned and control rats; in addition, sham-lesioned rats also spent less time in anogenital exploration and in female pursuit than controls. Conversely, the percent time spent in sniffing and rearing did not differ between sham-lesioned and control rats, but was in both groups significantly shorter than in nLOT-lesioned rats (Fig. 10d). All control and sham-lesioned rats exhibited mounting, but the latency to mount was about four times longer in sham-lesioned than in control rats. In contrast, none of the nLOT-lesioned rats exhibited mounting over the 10 min of testing (Fig. 10e).

nLOT-lesioned rats do not exhibit male–male aggressive behavior

We found a significant effect of treatment on the percentage of cumulative time spent in offensive ($F_{2,27} = 5.2$, $p < 0.001$) and in defensive ($F_{2,27} = 48.3$, $p < 0.001$) behaviors. Post-hoc analysis showed that nLOT-lesioned rats spent less time in offensive behaviors and more time in defensive behaviors than control and sham-lesioned rats (Fig. 11a). No significant differences were found between sham-lesioned and control rats. In offensive behaviors

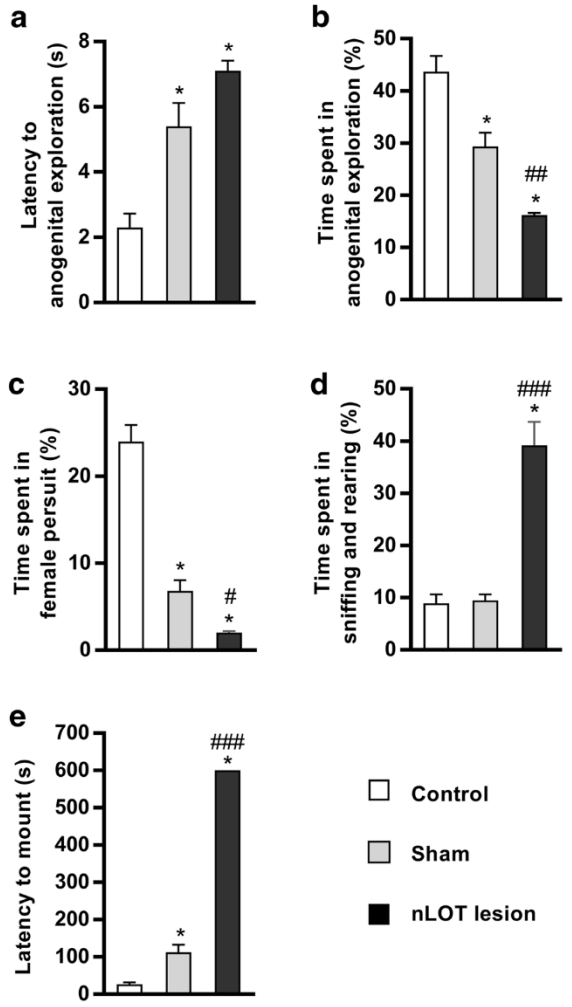

Fig. 10 nLOT-lesioned rats have impaired sexual behavior. Histograms show means + SEM values. **a** The latency to the first anogenital exploration of receptive females was significantly longer in nLOT- and sham-lesioned rats than in control. No differences were found between nLOT- and sham-lesioned rats. **b** The percentage of cumulative time over the duration of the test that nLOT-lesioned rats engaged in anogenital exploration was significantly shorter than in sham-lesioned and control rats. Sham-lesioned rats also spent significantly less time in anogenital exploration than controls. **c** The percentage of cumulative time over the duration of the test that nLOT-lesioned rats spent in female pursuit was significantly shorter than that spent by sham-lesioned and control rats. Sham-lesioned rats also spent significantly less time in female pursuit than controls. **d** The percentage of cumulative time over the duration of the test that nLOT-lesioned rats spent in sniffing and rearing behaviors was significantly shorter than that spent by sham-lesioned and control rats. No differences were found in these behaviors between sham-lesioned and control rats. **e** None of the nLOT-lesioned rats exhibited mounting over the 10 min of testing. Contrariwise, all sham-lesioned and control rats exhibited mounting, but the latency to mount was significantly longer in sham-lesioned than in control rats. $^{*}p < 0.001$ compared to control rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to sham-lesioned rats

(Fig. 11b), we found a significant effect of treatment on the percentage of cumulative time spent in attack ($F_{2,27} = 7.0$, $p < 0.01$), offensive upright ($F_{2,27} = 4.9$, $p < 0.05$) and lateral threat ($F_{2,27} = 5.8$, $p < 0.01$), but not in the keep down behavior ($F_{2,27} = 1.8$, n.s.). nLOT-lesioned rats spent less time in attack, offensive upright and lateral threat behaviors than control and sham-lesioned rats. No significant differences were found between sham-lesioned and control rats. When analyzing defensive behaviors (Fig. 11c), we also found a significant effect of treatment on the percentage of cumulative time spent in move away ($F_{2,27} = 24.6$, $p < 0.001$), submissive posture ($F_{2,27} = 25.0$, $p < 0.001$) and defensive upright ($F_{2,27} = 33.5$, $p < 0.001$). nLOT-lesioned rats spent more time in moving away, submissive posture and defensive upright behaviors than control and sham-lesioned rats. Once again, no significant differences were found between sham-lesioned and control rats.

As opposed to control and sham-lesioned rats, nLOT-lesioned rats did not attack and were not aggressive towards intruder males, and were more submissive, suggesting that nLOT lesions reduce male aggressive behavior.

nLOT-lesioned rats do not display cognitive alterations

The mean distances travelled by rats to find the submersed platform in the reference memory task of the Morris water maze are show in Fig. 12a. Repeated measures ANOVA revealed that rats of all groups progressively improved their ability to locate the hidden platform during the 14 days of acquisition ($F_{6,162} = 117.0$, $p < 0.001$). No differences between the groups were found, as shown by the absence of a significant main effect of treatment ($F_{2,27} = 1.5$, n.s.) and of treatment × trial blocks interaction ($F_{12,162} = 1.1$, n.s.). Behavioral analyses derived from the probe trial are shown in Fig. 12b, c. Two-way ANOVA showed that there was a significant effect of quadrant ($F_{1,54} = 398.4$; $p < 0.001$), but no significant effect of treatment ($F_{2,54} = 0.1$, n.s.) and no treatment × quadrant interaction ($F_{2,54} = 0.7$, n.s.). Rats of all groups spent more time in the target quadrant than in opposite quadrant. Moreover, the time spent in the target quadrant was similar for all groups, revealing that there were no differences among groups on the spatial strategy to search the escape platform during the probe trial. There were also no differences between groups in the number of times that rats crossed the former position of the platform ($F_{2,27} = 1.5$, n.s.; Fig. 12c). Rats of all groups rapidly learned to find the visible platform. The average distances swam over the eight trials to locate

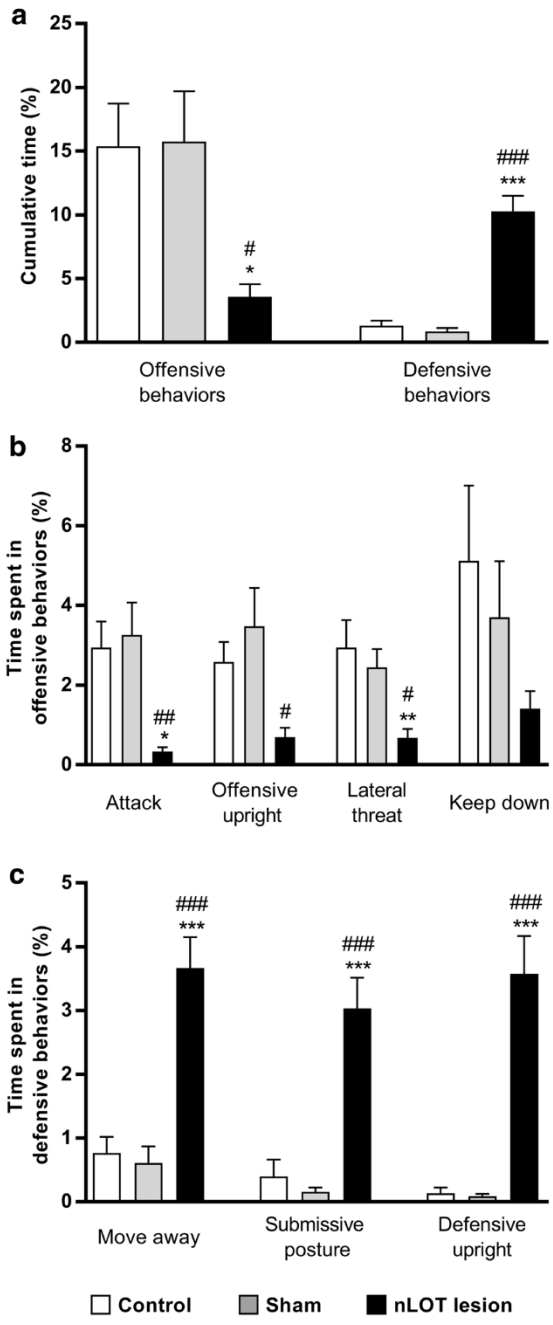

Fig. 11 nLOT-lesioned rats do not show aggressive behavior as revealed by the resident-intruder test. Histograms represent means + SEM values of the percentage of the cumulative time over the duration of the test that rats engaged in offensive and defensive behaviors. **a** nLOT-lesioned rats spent significantly less time in offensive behaviors and significantly more time in defensive behaviors than control and sham-lesioned rats. **b** nLOT-lesioned rats spent significantly less time in attack, offensive upright and lateral threat offensive behaviors than control and sham-lesioned rats. **c** Percentage of cumulative time that rats spent in moving away, submissive posture and defensive upright behaviors. nLOT-lesioned rats spent significantly more time in moving away, submissive posture and defensive upright behaviors than control and sham-lesioned rats. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to sham-lesioned rats

the platform position, expressed in cm (SEM), were 225 (58) for controls, 228 (80) for sham-lesioned rats and 206 (66) for nLOT-lesioned rats. No significant differences among the groups were found ($F_{2,27} = 0.3$, n.s.), showing that rats in all groups had similar sensorimotor abilities.

Lesions of the nLOT did not introduce any change in the immobility time evaluated in the forced swim test ($F_{2,27} = 0.3$, n.s.; Fig. 12d), which indicates that lesions of the nLOT do not interfere with the cognitive functions that underlie behavioral adaptation and survival (Molendijk and de Kloet 2015).

Discussion

The studies reported here were undertaken to determine the extent of functional and behavioral deficits resulting from lesions of the nLOT. Our results show that in male rats, the nLOT plays a role in the sense of smell and in innate behaviors, namely sexual behavior, aggression and predator avoidance. These deficits were observed in the absence of changes in locomotor activity, anxiety, fear, depression and cognitive functions.

nLOT-lesioned rats experienced a weight loss of about 8% during the first week post-surgery. This variation was also apparent in sham-lesioned rats, which indicates that the surgery itself and the stress inherent to it might be responsible for the reduced food ingestion and consequent body weight loss observed in both groups. These changes were transient and followed 1 week later by a progressive increase in food intake and body weights that, however, was insufficient to overcome the weight loss subsequent to surgery. Consequently, at the end of the experiments (i.e., 7 weeks later), control rats were significantly heavier than nLOT- and sham-lesioned rats. The lower body weights of rats in these groups were, in part at least, explained by their smaller amount of adipose tissue. This is consistent with the finding of significantly lower leptin levels in nLOT- and sham-lesioned rats as there is ample evidence that circulating leptin levels tend to parallel adipose tissue mass (Maffei et al. 1995). The nonexistence of differences in all these parameters between nLOT- and sham-lesioned rats indicates that the nLOT might not be especially relevant to the regulation of food intake or energy homeostasis, despite

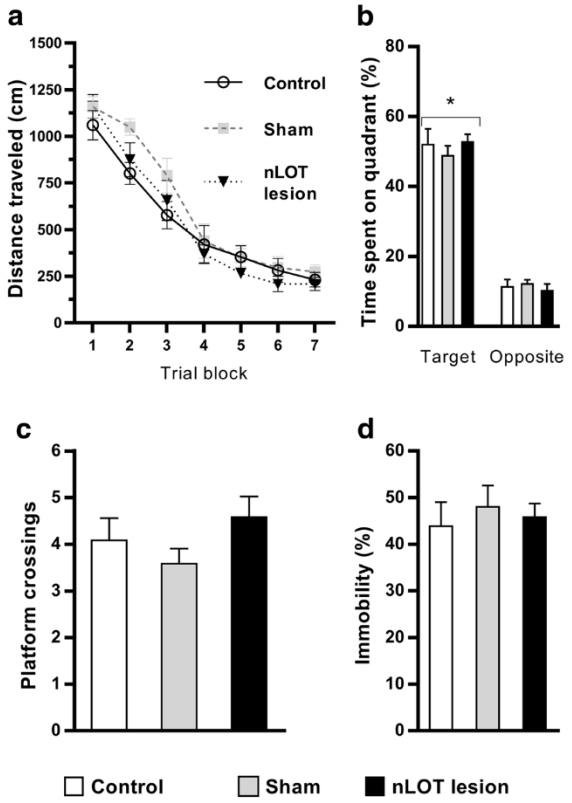

Fig. 12 nLOT-lesioned rats do not display cognitive alterations. **a**–**c** Morris water maze test. The graph in **a** shows the mean ± SEM total distances travelled (cm) to find the hidden platform for each block of four consecutive trials in the Morris water maze. There were no significant differences in acquisition performance between groups. The histogram in **b** shows mean + SEM values of the percentage of the cumulative time spent, over the duration of the test, on the quadrant where the hidden platform was located compared to the opposite quadrant. No significant differences were found between the three groups. The histogram in **c** shows mean + SEM number of platform crossings over the duration of the test. No significant differences were found among groups. **d** Forced swim test. The histogram shows the mean + SEM percentage of cumulative immobility time over the duration of the test. No differences were found between nLOT-lesioned, sham-lesioned and control rats. *$p < 0.001$ compared to the opposite quadrant

the fact that it expresses the long form of the leptin receptor Ob-Rb (Elmquist et al. 1998), contains melanocortin-expressing neurons (Saito et al. 2001) and projects directly or via multisynaptic pathways to brain regions involved in feeding behavior (Santiago and Shammah-Lagnado 2004).

Particularly impressive in our study was the finding that nLOT-lesioned rats had severe olfactory deficits with inability to detect and discriminate between odors. In the buried food test, nLOT-lesioned rats spent significantly more time to find the hidden cookie than sham-lesioned and control rats. This difference was not due to motor, motivational or other sensory deficiencies because with visible cues present (surface cookie) nLOT-lesioned rats performed the test as well as sham-lesioned and control rats. To better characterize the extent of olfactory impairment in nLOT-lesioned rats, we used the olfactory habituation/cross-habituation test. Control and sham-lesioned rats displayed decreasing durations of investigative behavior when repeatedly exposed to each of the five different odors tested, which is consistent with habituation. Conversely, nLOT-lesioned rats did not display such behavior in any of the odors tested. When a new odor was presented, the duration of investigative behavior was significantly longer in control and sham-lesioned rats, which indicates that they were capable of smelling the new odor (cross-habituation). However, that did not happen with nLOT-lesioned rats, suggesting that they have decreased olfactory sensitivity. The olfactory deficits detected in nLOT-lesioned rats were not consequent to the insertion of the stereotaxic needle as, in both tests, sham-lesioned rats behave similarly to controls. This indicates that the integrity of the nLOT is required for normal olfactory functions.

Odors elicit a variety of innate behaviors that are essential for the survival of the species, namely aversive responses to predator odors (reviewed in Canteras et al. 2015). Because these behaviors are observed in naïve animals, they are believed to be mediated by genetically determined and non-overlapping olfactory circuits (Malnic et al. 1999; Pacifico et al. 2012; Pérez-Gómez et al. 2015). Some nuclei/zones of the olfactory cortical group of the amygdala (Wernecke et al. 2015), and in particular the posterolateral cortical nucleus (Root et al. 2014) that receives spatially stereotyped projections from the main olfactory bulb (Pro-Sistiaga et al. 2007; Sosulski et al. 2011), seem to be essential for mediating approach and avoidance behaviors towards attractive and aversive odors, respectively. Since none of those studies has specifically examined the nLOT, we have addressed this subject herein by analyzing if lesions of the nLOT would interfere with the display of these behaviors, i.e., if nLOT-lesioned rats have, or not, the capacity to differentiate between appetitive and aversive odors. We compared several predator odors whose peripheral detection has been associated with different olfactory structures (main or accessory olfactory systems) or receptors (namely, different trace-amine associated receptors of the main olfactory epithelium, TAARs; Liberles and Buck 2006): PEA, which is present in carnivore urine and specifically activates TAAR4 (Ferrero et al. 2011; Dewan et al. 2013; Liberles 2015); IPA, a biogenic amine produced by leucine decarboxylation that is innately aversive to rodents and activates TAAR3 (Liberles 2015); TMT, a thiazole that is present in fox feces and activates the main olfactory epithelium (Root et al. 2014; Takahashi 2014; Pérez-Gómez et al. 2015); and CFO that primarily

activates the vomeronasal organ (Takahashi 2014; Pérez-Gómez et al. 2015). Because, as demonstrated in the posterolateral cortical nucleus of the amygdala, attractive and aversive odors activate different subsets of neurons (Root et al. 2014), and thus the behavioral effects they elicit are likely to be mediated by distinct neural projections, we have also tested the response of nLOT-lesioned rats to the attractive odorant 2PE. Control and sham-lesioned rats demonstrated attraction to 2PE and aversion to all predator odorants, as opposed to nLOT-lesioned rats that failed to show any attractive or avoidance behavior. These experiments demonstrate that nLOT lesions prevent the display of avoidance to predator odors, irrespective of the receptor or olfactory system activated by the odor. Since nLOT neurons do not seem to project to other cortical amygdaloid nuclei known to be involved in avoidance to predator odors, present data suggest that the integrity of the nLOT is required for the expression of this behavior. It must be mentioned here that although TMT induced avoidance behavior in control and sham-lesioned rats, we found no statistical significant differences between these groups when comparing the percentage of time spent investigating TMT and water. This was not related to the concentration of TMT used because the response was similar when the concentration was increased by five times (data not shown). In addition to the characteristics of the synthetic odorant used in this study, the possibility that the rat strain that we have used might have contributed for this finding cannot be ruled out, because as shown in several previous studies (Rosen et al. 2006; Staples and McGregor 2006; Staples et al. 2008; Staples 2010), Wistar rats seem to be less sensitive to TMT than other rat strains. Also, somehow intriguing in our study was the finding that CFO induced a robust avoidance behavior in control and sham-lesioned rats if one considers that the activation of the accessory olfactory system is traditionally thought of as requiring direct contact with the stimulus source. However, the demonstration that exposure to CFO significantly increases the number of FOS-positive cells in the main olfactory bulb (McGregor et al. 2004) raises the possibility that the odor from cat fur might contain volatile sensory cues that can be detected by the olfactory epithelium.

It is nowadays commonly accepted that volatile odors that function as pheromones and influence pheromone-driven behaviors are sensed and processed through both the main and the accessory olfactory systems (Keller et al. 2009; Brennan and Keverne 2015; Brignall and Cloutier 2015; Stowers and Kuo 2015). In rodents, the activation of the main olfactory system by conspecific volatiles seems to be important for social approach and mate recognition (for an overview, see Petrulis 2013; Baum and Cherry 2015). Thus, we next examined if nLOT lesions would interfere with the recognized attraction that sexually inexperienced male rats have to volatile odors from estrus females when compared to diestrus females or males (López et al. 1999; Portillo and Paredes 2004). Our results show that nLOT-lesioned rats have no preference for estrus female odors relative to diestrus female or male odors, indicating that they were not able to detect volatile estradiol-dependent female chemosignals or testosterone-dependent male chemosignals. This behavior contrasts with that observed in control and sham-lesioned rats that spent about twice the time investigating the odor stimulus of estrus over diestrus or male rats. These findings show that lesions of the nLOT interfere with the development of attraction to receptive female odors and, thus, presumably hinder mate recognition. Because volatile chemosignals are primarily sensed by the main olfactory epithelium (for a review, see Petrulis 2013), we can hypothesize that lesions of the nLOT disrupt the neural projections of the main olfactory system that mediate these behaviors.

In addition to receiving inputs from the main olfactory bulb, the nLOT also receives projections from the accessory olfactory bulb (Pro-Sistiaga et al. 2007; Gutiérrez-Castellanos et al. 2014). Since social investigation activates the accessory olfactory system (reviewed in Baum and Cherry 2015; Hashikawa et al. 2016) and this system has an important and complementary role to the main olfactory system (reviewed in Keller et al. 2009; Brennan and Keverne 2015; Brignall and Cloutier 2015; Stowers and Kuo 2015), we have also examined if nLOT-lesioned rats have abnormalities in male-specific behaviors in which the vomeronasal system seems to play an important role, that is, sexual behavior and aggression. Our results show that nLOT-lesioned rats display little interest towards sexually receptive females even when they can interact physically with them, as shown by the increased latency to anogenital exploration, the dramatic reduction in the time spent in anogenital exploration and in female pursuit, and the fourfold increase in the time spent in sniffing and rearing relative to sham-lesioned and control rats. Sham-lesioned rats also spent less time in anogenital exploration and in female pursuit than controls, and their latency to mount was longer than in controls. It is possible that the damage caused by the needle insertion might underlie the behavioral deficits observed in sham-lesioned rats and partially contribute for those displayed by nLOT-lesioned rats. Actually, the trajectory of the needle recurrently included the sublenticular portion of the substantia innominata, a brain region that in addition to being a component of the extended amygdala (McDonald 2003) and projecting to hypothalamic nuclei involved in male sexual behavior (Grove 1988), has been identified as potentially important for regulating social interest in rodents (reviewed in Hashikawa et al. 2016). However, lesions of the nLOT further aggravated the deficits in sociosexual behaviors

displayed by sham-lesioned rats, and in sharp contrast with sham-lesioned and control rats, who consistently mounted females, nLOT-lesioned rats failed to display any mounting behavior and, thus, to initiate copulatory behavior.

As expected, in the resident-intruder test, the percent time spent by resident control rats in offensive behaviors towards the intruders was significantly higher than that spent in defensive behaviors. Sham-lesioned rats behaved similar to control rats. Conversely, nLOT-lesioned rats spent approximately three times more time in defensive behaviors towards the intruder than in offensive behaviors. In addition, the time spent by nLOT-lesioned rats in offensive behaviors was just about one quarter of that spent by control and sham-lesioned rats in those behaviors. It is known that sexual and aggressive behaviors in males are influenced by the circulating levels of testosterone (Hull and Dominguez 2007; Albers 2012; Yang and Shah 2014). However, this is not a likely cause of the behavioral changes displayed by nLOT-lesioned rats because their serum concentrations of testosterone did not differ from those of sham-lesioned and control rats. Studies in male mice with complete loss of the main olfactory epithelium functions (Mandiyan et al. 2005; Wang et al. 2006) or chemical ablation of this epithelium (Keller et al. 2006) showed changes in chemoinvestigatory behavior, mounting and aggression similar to those found in nLOT-lesioned rats. Conversely, mutant male mouse strains with only partial defects in the main olfactory system and no general defects in olfaction display deficits in several social behaviors, including chemoinvestigatory preference and aggression, but not in mounting behavior (Matsuo et al. 2015). In light of these data, we can conclude that lesions of the nLOT interfere with the chemosensory processing mediated by the main and the accessory olfactory systems that is required for the display of copulatory and aggressive behaviors in male rats.

Because the behavioral alterations that we have detected in nLOT-lesioned rats might be linked to increased levels of anxiety-like behavior, we have assessed locomotor and exploratory activities in all groups of rats. We found no side effects of nLOT lesions on locomotor activity or general state of anxiety, as demonstrated by the absence of differences between groups in the tests performed in the open-field and elevated plus-maze, namely in the distances travelled in the outer and in the inner zones of the open-field as well as in the open arms, closed arms, and in the central square of the elevated plus-maze or in defecation and urination scores. The behavioral changes displayed by nLOT-lesioned rats were not also attributable to anhedonia because nLOT lesions had no effect on the sucrose preference test. Cognitive abilities evaluated in the Morris water maze were intact, suggesting that the hippocampal-dependent learning and memory are not affected by nLOT lesions. Likewise, nLOT lesions did not interfere with the cognitive functions that underlie stress coping and adaptation, as evaluated by the forced swim test, in which the mesoaccumbens dopaminergic circuit, which is under the control of the hippocampus and amygdala and receives inputs from the nLOT, plays an important role (Molendijk and de Kloet 2015; de Kloet and Molendijk 2016). Also contextual and cued fear conditioned learning and memory, in which one of the projection areas on the nLOT, the basolateral amygdala, plays a critical role (Kim and Jung 2006; Curzon et al. 2009), were unaltered in nLOT-lesioned rats. This was somehow surprising in view of the fact that odor was one of several sensory modalities that defined the context. However, and similar to other odors, nLOT-lesioned rats were unable to smell acetic acid, the odor used in contextual fear conditioning, irrespective of its concentration. Therefore, the normal level of contextual fear in nLOT-lesioned rats suggests that these rats may have increased reliance on visual and tactile cues at the expense of olfactory cues, a possibility that is supported by data from a recent study (Huckleberry et al. 2016) showing that changing only the floor of the test box is sufficient to render two contexts discriminable. The absence of changes in these behaviors shows that the consequences of bilateral lesions of the nLOT differ from those induced by bilateral olfactory bulbectomy because, in this experimental condition, anosmia and reduced sexual activity are associated to hyperactivity, anhedonia and marked cognitive decline (Song and Leonard 2005; Hendriksen et al. 2015).

To conclude, we describe here for the first time functional and behavioral effects of excitotoxic lesions of the nLOT in adult sexually naïve male rats. While some olfactory-related deficits might be expected due to the mixed chemosensory information it receives through both olfactory and vomeronasal projections, the extent and the nature of the effects of nLOT lesions were astounding. They included a complete loss of the sense of smell, with incapacity to identify and discriminate between odors. Possibly due to these effects on general olfactory abilities, nLOT lesions also prevented the display of innate odor-driven behaviors that are critical for species survival and reproduction. We do not know the reasons for the extent and severity of these effects, and we can only speculate at this point that, irrespective of the particular functional attributes of each component of the olfactory system, the normality of olfactory functions and olfactory-driven behaviors seems to require the integrity of all components of the olfactory system.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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III.

Chronic stress leads to long-lasting deficits in olfactory-guided behaviors, and to neuroplastic changes in the nucleus of the lateral olfactory tract

Chronic stress leads to long-lasting deficits in olfactory-guided behaviors, and to neuroplastic changes in the nucleus of the lateral olfactory tract

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Abstract

A recent study reported that the integrity of the nucleus of the lateral olfactory tract (nLOT) is required for normal olfaction and for the display of odor-driven behaviors that are critical for species survival and reproduction. In addition to being bi-directionally connected with a key element of the neural circuitry that mediates stress response, the basolateral nucleus of the amygdala, the nLOT is a potential target for glucocorticoids as its cells express glucocorticoid receptors. Herein, we have addressed this hypothesis by exploring, first, if chronic variable stress (CVS) disrupts odor detection and discrimination, and innate olfactory-driven behaviors, namely predator avoidance, sexual behavior and aggression in male rats. Next, we examined if CVS alters the nLOT structure and if such changes can be ascribed to stress-induced effects on the activity of the main output neurons, which are glutamatergic, and/or of local GABAergic interneurons. Finally, we analyzed if the stress-induced changes are transient or, conversely, persist after cessation of CVS exposure. Our data demonstrate that CVS leads to severe olfactory deficits with inability to detect and discriminate between odors and to innately avoid predator odors. No effects of CVS on sexual and aggressive behaviors were observed. Results also showed that CVS leads to somatic hypertrophy of pyramidal glutamatergic neurons, which likely results from neuronal disinhibition consequent to the loss of inhibitory inputs mediated by GABAergic interneurons. Most of the CVS-induced effects persist beyond a 4-week stress-free period, suggesting long-lasting effects of chronic stress on the structure and function of the olfactory system.

Keywords: Chronic unpredictable stress; Cortical amygdala; Nucleus of the lateral olfactory tract; Calcium-binding proteins; GABA; Olfaction; Sexual behavior; Aggression; Stereology.

1. Introduction

Prolonged exposure to stress affects virtually all bodily systems and increases the vulnerability to numerous health problems (Chrousos, 2009). In particular, it has been implicated in the pathophysiology of neuropsychiatric disorders such as post-traumatic stress, major depression and anxiety due to the adverse nature of its cumulative effects on neuroplasticity, which ultimately lead to cognitive and behavioral deficits (Chrousos, 2009; Kim et al., 2015; Lupien et al., 2009; Roozendaal et al., 2009). The effects of prolonged stress have been mostly studied, and are more apparent, in brain regions that have been implicated in neuropsychiatric disorders and are sensitive to stress hormones, i.e., the hippocampus, prefrontal cortex and amygdala (reviewed in Radley et al., 2015). Interestingly, the pattern of the stress-induced structural and functional alterations in the amygdala differs completely from that observed in the hippocampus and prefrontal cortex. Moreover, and also in contrast to these regions, different stress paradigms and different durations of stress exposure provoke distinct structural and functional responses in the amygdala (reviewed in Lupien et al., 2009; Radley et al., 2015; Wilson et al., 2015). Also important is that, in contrast to the hippocampus, the morphological and functional changes in the amygdala last for weeks after termination of chronic stress (Sousa et al., 2000; Vyas et al., 2004).

Most studies addressing the effects of stress on the amygdala have focused on the basolateral complex and central nucleus. The basolateral complex receives the majority of the inputs to the amygdala. Here, the information is processed before being transmitted to the central nucleus, which gives rise to projections to the behavioral, autonomic and endocrine effector systems (reviewed in McDonald, 1998). Afferents to the basolateral complex derive mostly from cortical areas and convey sensory inputs of all types (McDonald, 1998). Considering the recognized role of olfaction in emotional

and cognitive processes (Song and Leonard, 2005; Yuan and Slotnick, 2014), it is intriguing that the flow of olfactory information to the basolateral complex is not direct, but instead mediated by afferents originating in the cortical pallial amygdala, a target area of the main olfactory bulb and piriform cortex (McDonald, 1998). In this context, the nucleus of the lateral olfactory tract (nLOT) is of particular interest as it projects strongly, and in a topographically organized way, to the basolateral amygdaloid complex from where it receives reciprocal connections. Moreover, it also projects to the prefrontal cortex (Jolkkonen et al., 2001; Luskin and Price, 1983; McDonald, 1991; Price, 1973; Santiago and Shammah-Lagnado, 2004), which is bi-directionally connected the basolateral amygdaloid complex (McDonald, 1998). Last but not the least, it is known that lesions of the nLOT are associated with a complete loss of the sense of smell and disruption of olfactory-guided behaviors (Vaz et al., 2017).

Due to the reasons mentioned above and to likelihood that the nLOT is a stress-sensitive nucleus, as it possesses a large number of cells expressing glucocorticoid receptor mRNA and protein (Morimoto et al., 1996), we thought of particular interest to examine whether the nLOT displays structural and neurochemical alterations that might contribute to explain the functional and behavioral effects of chronic stress, particularly those in which the olfactory system plays a relevant role. To accomplish this, we have used a chronic variable stress paradigm and examined its effects on olfactory functions and innate olfactory-driven behaviors, namely predator avoidance, sexual behavior and aggression. In addition, we have estimated the number and size of nLOT neurons and of its different classes of interneurons, and quantified the local levels of GABA. Finally, to analyze whether the stress-induced alterations would be reversible or permanent, we performed the same studies 4-7 weeks after the end of the stress protocol.

2. Material and Methods

2.1. Animals and treatments

The experiments were conducted in male Wistar rats (Charles River, France). After acclimation to laboratory conditions for at least 1 week, rats were housed in groups of 3 and maintained in standard environmental conditions (12-h light/dark cycles with lights on at 0700 h, ambient temperature of 21 ± 1ºC, 45 ± 5% relative humidity) with *ad libitum* access to food and water, unless specifically noted. All experiments were carried out in accordance with the guidelines of the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act nº113/13, and approved by ORBEA, the internal committee of the Faculty of Medicine, University of Porto (Portugal). At 3 months of age (Fig. 1), rats were randomly assigned to a group receiving chronic variable stress (CVS; n = 18), also referred to as chronic unpredictable, mild or intermittent stress (Willner, 2017), to a stress-recovery group (n = 18) or to a control group (n = 18). Starting at 4 months of age, rats of the stressed group were exposed daily, over 4 weeks, to one of four stressors: i.p. injection of hypertonic saline (9% NaCl; 1.0 ml/100 g), overcrowding (1 h), restraint (30 min) and placement on a vibrating/rocking platform (1 h). To maximize unpredictability, stressors were applied at various times during the light phase. Rats of the recovery group started to be exposed to the same CVS paradigm 4 weeks before, i.e., about the age of 3 months and, thereafter, they were left undisturbed for an additional 4 weeks. At the end of the experiments, rats of both groups were submitted to a series of behavioral studies and then killed. Control rats were handled daily before being submitted to the same behavioral evaluation. During the experiments, rats were weighed every morning. Post-mortem weights of adrenal glands and thymus, which were quickly removed after

euthanasia, were also recorded as indicators of the efficacy of the stress protocol. Testis were also collected and immediately weighed. For determination of corticosterone levels, blood samples were collected from the dorsal tail vein at the end of CVS exposure, directly from the heart immediately before perfusion or from the trunk after decapitation. The last samples were also used for measurement of testosterone concentrations.

2.2. Behavioral studies

All tests were performed during the standard light phase, starting at 1400 h, except for sexual behavioral and aggression tests that were started 1 h after the beginning of the dark phase. Three days after the end of the experimental periods, rats (n = 18/group) were submitted to the buried food test followed by the olfactory habituation/cross-habituation test (Fig. 1). Then, the three main groups were divided in two independent cohorts. One cohort (n = 9/group) was submitted to open-field and elevated plus-maze before being tested for olfactory preference tests. The second cohort (n = 9/group) was tested for sucrose preference, and sexual and aggressive behaviors. All tests were applied with at least 1-day inter-trial intervals. In order to maintain a constant lag time between the tests performed in stressed and in recovery rats, the sequence of the tests was the same for all main groups. Rats were killed 3-4 days after the end of behavioral testing (Fig. 1).

2.2.1. Open-field test.

To assess general exploratory locomotion and anxiety-like behaviors, an open-field apparatus that consisted of a white acrylic arena (100 × 100 × 40 cm) was used. As previously described (Cardoso et al., 2016; Vaz et al., 2017), the inner zone was defined

as 60 × 60 cm square in the center of the arena, leaving a 20 cm broader zone (the outer zone) on each side. The rat was placed in a corner of the apparatus and tested during 5-min sessions. Distances traveled in the outer and in the inner zones were measured using a computerized video-tracking system (EthoVision XT 8.5, Noldus, The Netherlands). At the end of each session, the number of fecal boli deposited was counted, and the urine deposited was collected using a filter paper. The difference between the weight (in g) of the paper before and after collecting the urine was considered as a measure of the amount of urine deposited during the session. The floor of the apparatus was then thoroughly cleaned and dried.

2.2.2. Elevated plus-maze.

To further evaluate general exploratory and anxiety-like behaviors, we used an elevated plus-maze apparatus consisting of a black acrylic cross with 2 opposite open and 2 opposite closed arms (50 × 12 cm, enclosed by 50-cm high walls) joined by a common central square (12 × 12 cm). The test rat was placed on the central square facing one of the closed arms and allowed to explore the apparatus for 5 min. The behavior of the rat was recorded and analyzed using a computerized video-tracking system (EthoVision XT 8.5, Noldus). The percentages of time spent and the distances traveled by rats in the open arms, closed arms and central square were computed. At the end of each session, the number of fecal boli and the amount of urine were recorded. The apparatus was then thoroughly cleaned and dried.

2.2.3. Sucrose preference test.

To assess possible anhedonia (i.e., decreased sensitivity to reward), which is generally associated to depression, rats were subjected to the sucrose preference test.

One day before the test, rats were acclimated to the two-bottle configuration and to sucrose taste. During the 4 consecutive days, rats were given the choice to drink from 2 bottles placed side-by-side, one containing water and the other a 2% sucrose (Sigma–Aldrich) solution. The total amount of liquid consumed by rats was measured daily and fresh solutions were prepared. To avoid possible effects of bottle side bias, the position of the bottles was switched every 12 h. Sucrose preference was calculated by taking the proportion of sucrose solution consumed over the total amount of liquid consumed during the 4-day testing period.

2.2.4. Buried food test.

The test was performed, as described before (Vaz et al., 2017; Yang and Crawley, 2009), to assess the ability of rats to smell volatile odors and their tendency to use olfactory cues for foraging. For odor familiarization, a highly palatable cookie was placed in the test chamber during 2 consecutive days before the test, and confirmed that it was consumed by the rat. The chamber consisted of a clean standard plastic cage (44 × 34 × 20 cm) with a 5 cm layer of new bedding. After 18 h of food deprivation, the rat was placed in the chamber for 10 min to acclimate. Then, the rat was removed from the cage and a cookie was buried in the bedding, approximately 2 cm beneath the surface, at a random location. The bedding surface was smoothed out and the rat was re-introduced into the cage. The time spent to locate the buried cookie was recorded. The maximum test time allowed was 900 s. Two hours later, a surface cookie test was performed. This test was set up in the same way as the buried food test, but the cookie was placed on the surface of the bedding instead of being buried.

2.2.5. Olfactory habituation/cross-habituation test.

To assess the ability of rats to detect and discriminate between a familiar and a novel odor, we performed the test as previously described (Vaz et al., 2017; Yang and Crawley, 2009). Prior to testing, the rat was allowed to acclimate for 10 min to a clean cage (38 × 24 × 20 cm), in which a plastic tube applicator (10 × 0.5 cm) containing a rectangular filter paper inside (3 × 1 cm) was inserted through the water bottle hole. We used this kind of applicator to avoid direct contact with the odor stimulus. The test consisted of sequential presentations of water and 4 different odors in 3 consecutive trials of 2 min, with 1-min inter-trial intervals. The sequence used was water, 2 nonsocial odors (100 µl lemon extract, 1:10 dilution in distilled water, and 100 µl strawberry extract, 1:10 dilution in distilled water) and 2 different social male odors (100 µl of social odor solution). Each social odor was obtained by swabbing, with a cotton tip, the bedding of 2 different cages with male rats and diluting it in a glass vial with water and with 100 µl of urine collected from each of the males in those cages. The cumulative time spent sniffing the odorant in each presentation was quantified with a stopwatch, within the 2-min time period. To better differentiate between poor and fair odor discrimination, we calculated the cross-habituation index as the difference between the duration of investigation of all new odors and trial 3 of the odor presented immediately before (Wesson et al., 2010).

2.2.6. Olfactory preference tests.

Rats were allowed to habituate to the environment of the test room and to the open-field arena during 10 min for 2 d prior to testing. Then, they were submitted to a series of single odorant tests and, afterwards, to one triple odorant test. Rats were exposed to one stimulus per day. They were naïve to each odor (as the odors tested were

different from those used in the habituation/cross-habituation test) and were tested only once for each odor. In the single odor test, the open-field arena contained Petri dishes in each corner, with only one Petri dish containing the odor stimuli. In the 2 habituation trials, the conditions were the same of the experimental test, except that no odor was used. Exposure to the odor was done by placing a 3.5 cm covered Petri dish containing a piece of filter paper (2 × 2 cm) impregnated with the odor stimuli in one corner of the open-field apparatus. As already done in several studies (Dewan et al., 2013; Vaz et al., 2017), the top of Petri dish was perforated to allow odorants to escape and to prevent direct nasal contact. One Petri dish containing a filter paper without any odor was placed in each of the other corners of the open-field arena. Because animals were tested in the same open-field arena to all odors, each of these odors was introduced in a pseudo-random corner of the arena to avoid any corner bias. Then, the test rat was placed in the center of the arena and allowed to freely explore the apparatus during 15 min. Sessions were recorded using a computerized video-tracking system (EthoVision XT 8.5, Noldus) and later analyzed. The time spent by the rat actively investigating the corner where the odor was delivered was recorded and compared with the time rats spent investigating water. The odorants tested, chosen because their peripheral detection has been associated with different olfactory structures (main or accessory olfactory systems; Baum and Cherry, 2015) or receptors, namely different trace-amine associated receptors of the main olfactory epithelium (TAARs; Liberles and Buck, 2006), were: 2-phenylethanol (2PE, 50% in water), an aromatic alcohol with a rose scent used in the cosmetics, fragrance and food industries allowing to selectively probe the olfactory system (Kim et al., 2014); 2-phenylethylamine (PEA, 50% in water), which is present in carnivore urine and specifically activates TAAR4 (Dewan et al., 2013; Ferrero et al., 2011; Liberles, 2015); isopentylamine (IPA, 10% in water), a biogenic amine produced

by leucine decarboxylation that is innately aversive to rodents and activates TAAR3 (Liberles, 2015), and cat fur odor (CFO), which primarily, but not exclusively (McGregor et al., 2004), activates the vomeronasal organ (Pérez-Gómez et al., 2015; Takahashi, 2014). Chemicals were all purchased from Sigma–Aldrich. Cat fur was collected from a domestic adult male cat.

For the triple odor exposure, each rat was simultaneously presented with urine from group-housed testes-intact male rats, females in behavioral estrus and diestrus female rats. Urine was collected using metabolic cages. Urine from each group was pooled, aliquoted and stored at -80°C until use. All the procedures were identical to those in the single odor test, except that in this test 3 odors and water were presented simultaneously, one in each corner of the open-field cage. Then, the test rat was placed in the center of the arena and allowed to freely explore for 15 min. The time spent actively investigating each corner of the open-field was calculated. Sessions were recorded using a computerized video-tracking system (EthoVision XT 8.5, Noldus) and later analyzed.

2.2.7. Sexual behavior.

Rats were observed in pre-copulatory behaviors and mounting in 3 assays with 4-days inter-trial intervals. On each trial, the test rat was placed into the cage and allowed to acclimate for 10 min. A sexually experienced receptive female rat was then introduced into the cage. Females were brought into behavioral estrus by subcutaneous injections of 10 μg of estradiol benzoate 52 h before the test, followed by 500 μg of progesterone 4-6 h before testing. Sexual behavior was recorded with a video camera during a 10-min period. The latency to the first anogenital exploration and to the first mounting/intromission was recorded from the time the female entered the cage. The

cumulative duration of anogenital exploration and the mounting frequency (number of mounts per minute) were also determined.

2.2.8. Aggressive behavior.

Male aggression was assessed by the resident/intruder assay (Leypold et al., 2002). Rats in all groups were single-housed and maintained in its home cage without changing of the bedding for the previous 5 days. A group-housed, sexually and aggressively inexperienced and unfamiliar adult rat, lighter than the test rat, was introduced into the home cage of the test rat. The tests were video-recorded over 15 min, and later analyzed to determine the duration of offensive behaviors (chase, attack, offensive upright, lateral threat, keep down) and defensive behaviors.

2.3. Brain tissue preparation

At the end of behavioral testing, stressed, recovery and control rats were divided in 3 groups of 6 rats each: one group was used for the estimation of general morphometric parameters of the nLOT, a second group for immunocytochemical studies, and the third group for biochemical studies. Three to 4 days after behavioral testing (Fig. 1), rats were anesthetized with sevoflurane (SevoFlo, Abbott Laboratories Ltd, Maidenhead, UK) and killed, between 1900 and 2000 h, and blood samples were collected for hormone measurements.

Rats used for the estimation of general morphometric parameters of the nLOT were killed by transcardiac perfusion of a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (PB), at pH 7.2. The brains were removed from the skulls, coded, post-fixed for 30 d in fresh fixative, transected rostrally through the anterior border of the optic chiasm and caudally at the

level of the anterior limit of the mammillary bodies, and embedded in glycolmethacrylate (for details see West et al., 1991). The blocks were then serially sectioned in the coronal plane, at a nominal thickness of 40 µm, using a Jung Multicut microtome. Sections were mounted serially and stained with a modified Giemsa solution (West et al., 1991).

Rats used for immunohistochemical studies were perfused transcardially with 150 ml of 0.1 M PB, pH 7.6, for vascular rinse, followed by 250 ml of a fixative solution containing 4% paraformaldehyde in PB, at pH 7.6. The brains were removed from the skulls, coded, immersed for 1 h in the same fixative, and maintained overnight in a solution of 10% sucrose in PB, at 4ºC. After trimming away the frontal and occipital poles, the hemispheres were separated by a mid-sagittal cut. The blocks of tissue thus obtained were placed on a vibratome and serially sectioned in the coronal plane at 40 µm through the nLOT. Sections were collected in phosphate-buffered saline (PBS) before being transferred into de Olmos cryoprotectant solution, where they were stored at -20°C until further processing. Sections of the right hemisphere were alternately allocated for calretinin (CR) or calbindin D-28K (CB) immunostaining, whereas those of the left hemisphere were used for immunocytochemical detection of parvalbumin (PV). Sections to be immunostained were washed twice in PBS and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. For PV, CB and CR immunohistochemistry, sections were immersed, before incubation with the primary antibody, in a 5% solution of horse normal serum (Vector Laboratories, Burlingame, CA, USA) in PBS for 30 min at room temperature. Afterwards, they were incubated for 72 h, at 4ºC, with the primary antibody against PV (PV235, Swant, Switzerland; 1:5000), CB (CB300, Swant; 1:5000) or CR (CR6B3, Swant; 1:5000). The secondary antibody used was a biotinylated horse anti-mouse antibody (Vector; 1:400).

Afterwards, sections were all treated with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector; 1:800). In the last two steps, the incubation was carried out, at room temperature, for 1 h. Sections were finally incubated for 10 min in 0.05% diaminobenzidine (DAB; Sigma–Aldrich) to which H_2O_2 was added to a final concentration of 0.01%. Both the primary and the secondary antibodies were diluted in PBS to which 0.5% Triton X-100 was added. Between different incubations, sections were rinsed in PBS to which 0.5% Triton X-100 was added for at least 30 min. Sections were mounted on gelatin-coated slides, air-dried, dehydrated in a series of ethanol solutions (50%, 70%, 90%, and 100%), cleared in xylol and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). To prevent variability in staining, sections from the experimental and control groups were processed in parallel. To control for nonspecific binding, control sections were incubated as described above, but without the primary antibody; no immunostaining was observed in these sections.

Rats used for biochemical studies were killed by decapitation. Brains were quickly removed from the skulls and carefully dissected over a cold Petri dish placed on ice in order to isolate the nLOT. The tissue samples were weighed, frozen immediately in liquid nitrogen and stored at -80°C until analysis.

2.4. Hormone assays

After centrifugation, the serum was collected into aliquots and stored at -80°C until hormone analysis. Testosterone was determined by enzyme-linked fluorescent assay using a VIDAS Testosterone Kit (VIDAS Testosterone II, Reference 414320, bioMérieux SA, Marcy l'Etoile, France) and a VIDAS analyzer (bioMérieux SA). According to the manufacturer, the assay has a measurement range of 0.1-13 ng/ml. The analyzer was calibrated and operated in accordance with the manufacturer's instructions.

All samples were tested in singlicate. Corticosterone concentrations were measured with a commercial ELISA kit (ab10882 - Corticosterone ELISA kit, abcam, Cambridge, UK) following the manufacturer's recommendations. All samples were analyzed in duplicate.

2.5. Stereological analyses

The delineation of the nLOT and of its layers in Giemsa-stained sections was done based on cytoarchitectonic criteria already described (McDonald, 1983; Millhouse and Uemura-Sumi, 1985; Price, 1973; Vaz et al., 2016). The estimates of layer volumes and total neuron numbers were obtained separately for layers 1-3. Due to the small number of neurons in layer 1 (Vaz et al., 2017), neuronal volumes were only estimated in layers 2 and 3. Stereological estimations were all performed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage and an object rotator (Olympus, Albertslund, Denmark). A computer fitted with a framegrabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor. Neurons were counted and measured at final magnification of 2000× at the screen level, using a NA 1.40 ×100 oil-immersion objective lens.

For the estimation of layer volumes and total neuron numbers, all Giemsa-stained sections containing the nLOT were used. Layer volumes were estimated by applying the principle of Cavalieri and point counting techniques (Gundersen and Jensen, 1987; Madeira et al., 1997, 1995), at a final magnification of 100× at the screen level, using a grid of test points in which the area per point was 9.2 μm^2. On average, 300 points were counted per animal in layers 1 and 2, and 150 in layer 3; the coefficient of error (CE) of the estimates was, on average, 0.03 for layer 1 and 0.02 for layers 2 and 3. The volume of each layer was calculated from the total number of points that fell on that layer and

the section thickness. The volume of the nLOT was computed as the sum of the volumes of the individual layers.

The total number of neurons in each nLOT layer was estimated by applying the optical fractionator method (Madeira et al., 1997, 1995; West et al., 1991). In each section, the fields of view were systematically sampled using step sizes of 90 μm (layer 1), 120 μm (layer 2) or 60 μm (layer 3) in the *x and y* axes; the disectors had a counting frame area of 4953 μm^2 (layer 1), 693 μm^2 (layer 2) and 1189 μm^2 (layer 3), and a fixed depth of 10 μm. The average number of neurons counted was 200 (layer 1) and 235 (layer 2) and 270 (layer 3). The mean CE of the estimates, calculated as described by Gundersen et al. (1999), was, on average, less than 0.08. The total number of neurons in the nLOT was computed as the sum of the total number of neurons in layers 1-3.

The mean somatic volume ($\bar{V}_N$) of nLOT neurons was estimated on alternate sections by applying the optical rotator (Leal et al., 1998; Tandrup et al., 1997). Neurons used for measurements were selected with optical disectors, as described above. Measurements of intersections between the cell membrane and the spatial line grid were performed using a two-grid line and two focal planes per each cell. The mean CE of the estimates, calculated as described by Gundersen and Jensen (1987), was 0.05.

Due to the relatively small number of interneurons in the nLOT, the total number of PV-, CB- and CR-immunoreactive neurons was estimated without taking into account their cell layer location. Total numbers were estimated by using the optical disector method, as follows. In each section, the fields of view were systematically sampled using step sizes of 110 μm in the *x* axis, and 80 μm in the *y* axis; the disector had a counting frame area of 7924 μm^2 at the tissue level and a fixed depth of 10 μm. Neurons were considered as staining positively for PV, CB or CR when they had a brown-stained perikaryal cytoplasm outlining a relatively unstained nucleus. The

average number of PV-, CB- and CR-immunostained interneurons counted was 180, 120 and 170, respectively. The mean CE of the estimates was 0.07, 0.09 and 0.08, respectively.

2.6. Quantification of GABA in the nLOT

Tissue concentrations of GABA were determined using an Elisa kit for GABA (BA E-2500, LDN Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's instructions.

2.7. Statistical analyses

Behavioral and hormonal results are expressed as mean ± SEM and morphological data as mean ± SD. Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA). Repeated measures analysis of variance (ANOVA) was used to analyze data on olfactory habituation/cross-habituation (independent variables: water, lemon, strawberry, social 1, social 2, treatment; dependent variable: time spent sniffing the odor). Two-way ANOVA was used for analysis of data obtained in the buried food test (independent variables: cookie location, treatment; dependent variable: time spent to find the cookie), open-field (independent variables: zone, treatment; dependent variables: distance traveled), elevated plus-maze (independent variables: zone, treatment; dependent variables: distance traveled, time spent), sucrose preference test (independent variable: type of drink, treatment; dependent variables: liquid intake) and aggressive and defensive behaviors (independent variable: type of behavior, treatment; dependent variable: time spent in offensive, defensive behaviors). A one-way ANOVA was used to analyze the effect of treatment on the total distances travelled in the open-field and elevated plus-

maze, number of fecal boli and grams of urine, cumulative time spent by rats sniffing each odor over the 3 trials of the olfactory habituation/cross-habituation test, cross-habituation index, offensive behaviors (time spent in chase, attack, offensive upright, lateral threat and keep down), sexual behavior (latency to the first anogenital exploration, latency to the first mounting/intromission, cumulative duration of anogenital exploration and mounting frequency), body weight gain, organ weights, hormone concentrations, and stereological data. Data obtained in the single odor test applied to examine olfactory preference, compared to water, was analyzed using two-way ANOVA (independent variables: 2PE, PEA, IPA, CFO, treatment; dependent variable: time spent in odor corner), whereas the triple odor test was analyzed separately for each treatment group by one-way ANOVA (independent variables: water, non-receptive female, receptive female and male odors; dependent variable: time spent in the odor corners). Whenever appropriate, ANOVAs were followed by Tukey highest signification difference (HSD) post-hoc comparisons. Effect size comparisons were performed for ANOVA (partial eta squared, η^2_p) and post-hoc tests (Cohen's d, *d*). A $\eta^2_p > 0.06$ was considered to represent a medium effect size and $\eta^2_p > 0.14$ was considered to represent a large effect size. A Cohen's $d > 0.8$ indicates a large effect size. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Effects of CVS and subsequent recovery on behavioral data

3.1.1. CVS provokes anxiety-like behaviors that are attenuated by recovery

We measured anxiety-like behavior in the open-field (Fig. 2 A, D) and in the elevated plus-maze (Fig. 2 B-D). ANOVA showed significant main effects of treatment

($F(2,48) = 5.49$, $p = 0.007$, $\eta^2_p = 0.186$) and zone ($F(1,48) = 604.06$, $p < 0.0001$, $\eta^2_p = 0.926$) on the distances travelled in the outer and inner zones of the open-field; a significant treatment × zone interaction was also found ($F(2,48) = 5.86$, $p = 0.005$, $\eta^2_p = 0.196$). Rats of all groups travelled significantly smaller distances in the inner zone than in the outer zone of the open-field (Fig. 2A). The distances travelled in the outer zone were significantly smaller in stressed than in control rats, whereas in recovery rats they did not significantly differ from either stressed or control rats. ANOVA applied to data obtained in the elevated plus-maze showed that the percentage of time spent in the different zones of the elevated plus-maze and the distances travelled in each of the zones were significantly influenced by zone ($F(2,72) = 298.07$, $p < 0.0001$, $\eta^2_p = 0.892$, and $F(2,72) = 367.76$, $p < 0.0001$, $\eta^2_p = 0.911$, respectively), but not by treatment ($F(2,72) = 0.05$, $p = 0.951$, $\eta^2_p = 0.001$ and $F(2,72) = 2.78$, $p = 0.069$, $\eta^2_p = 0.072$, respectively); however, a significant treatment × zone interaction on the time spent in the different zone of the maze was found ($F(4,72) = 4.22$, $p = 0.004$, $\eta^2_p = 0.19$). Rats of all groups spent more time (Fig. 2B) and travelled longer distances (Fig. 2C) in the closed arms than in the central square and opens arms. Moreover, stressed rats spent significantly more time in the closed arms than control rats, whereas the time spent by recovery rats in those arms was intermediate between that of stressed and control rats and did not significantly differ from both. No significant differences between groups in the distances travelled in the different regions of the elevated plus-maze were found. There were no significant effects of treatment on the defecation and urination scores (Fig. 2D) obtained either in the open-field ($F(2,24) = 0.34$, $p = 0.716$, $\eta^2_p = 0.027$ and $F(2,24) = 0.10$, $p = 0.902$, $\eta^2_p = 0.009$ respectively) or in the elevated plus-maze ($F(2,24) = 0.02$, $p = 0.985$, $\eta^2_p = 0.001$ and $F(2,24) = 0.19$, $p = 0.827$, $\eta^2_p = 0.016$ respectively).

To assess general locomotor activity, we have also calculated the total distances travelled in the open-field and elevated plus-maze. The distances, expressed in cm (SEM), travelled in the open-field were 2854 (144) for control rats, 2058 (145) for stressed rats and 2439 (208) for recovery rats. In the elevated plus-maze, the total distances were 1320 (76), 1310 (64) and 1120 (49), respectively. There was a significant influence of treatment in the total distances travelled in the open-field ($F(2,24) = 5.57$, $p = 0.010$, $\eta^2_p = 0.317$), but not in the elevated plus-maze ($F(2,24) = 3.13$, $p = 0.062$, $\eta^2_p = 0.207$). CVS significantly decreased the total distance travelled in the open-field ($p = 0.008$, $d = 1.832$). The total distances travelled by recovery rats were intermediate between those of control and stressed rats, but the differences were not statistically significant.

Together, these data indicate that CVS increases anxiety-like behaviors and decreases locomotor and exploratory activities, and that this is not fully reversed 6 weeks after the last stress exposure.

3.1.2. CVS causes transient depressive-like behaviors

We found significant effects of treatment ($F(2,48) = 12.03$, $p < 0.0001$, $\eta^2_p = 0.334$) and type of drink ($F(1,48) = 347.98$, $p < 0.0001$, $\eta^2_p = 0.879$) on the sucrose preference test; a significant treatment × type of drink interaction was also found ($F(2,48) = 11.20$, $p < 0.0001$, $\eta^2_p = 0.318$). As expected, rats in all groups drank a significantly greater amount of sucrose solution than regular water (Fig. 3). Sucrose preference was significantly reduced in stressed rats, but returned to control levels at least 5 weeks after the end of stress exposure. These findings indicate that CVS increases anhedonia and depressive-like responses and that these effects are transient.

3.1.3. CVS provokes enduring olfactory deficits

As shown in Figure 4A, there was a significant influence of treatment ($F(2,102) = 7.08$, $p = 0.0013$, $\eta^2_p = 0.122$) and cookie location ($F(1,102) = 96.53$, $p = 0.0001$, $\eta^2_p = 0.486$) on the latency to find the cookie; a significant treatment × cookie location interaction ($F(2,102) = 6.96$, $p = 0.0015$, $\eta^2_p = 0.12$) was also found. The average latency to find the hidden cookie was significantly longer in stressed and recovery rats than in controls. No statistical significant difference was found between stressed and recovery rats. There were no differences among groups in the time spent to find the visible cookie.

In the olfactory habituation/cross-habituation test (Fig. 4B), repeated measures ANOVA showed that rats in all groups responded to the sequential presentation of the five odors ($F(14,714) = 16.78$, $p < 0.0001$, $\eta^2_p = 0.248$), and that there was a significant effect of treatment ($F(2,51) = 15.97$, $p < 0.0001$, $\eta^2_p = 0.385$) in the amount of olfactory investigation of the different odors. The analysis of individual odors showed that treatment significantly influenced the response to lemon ($F(2,51) = 11.27$, $p < 0.0001$, $\eta^2_p = 0.306$), strawberry ($F(2,51) = 6.11$, $p = 0.004$, $\eta^2_p = 0.193$), social 1 ($F(2,51) = 11.36$, $p < 0.0001$, $\eta^2_p = 0.308$) and social 2 ($F(2,51) = 9.87$, $p < 0.0001$, $\eta^2_p = 0.279$) odors, but not to water ($F(2,51) = 3.10$, $p = 0.054$, $\eta^2_p = 0.108$). Post-hoc analysis of these data revealed that stressed and recovery rats spent significantly less time sniffing lemon ($p < 0.0001$, for both groups, $d = 1.201$ for stress and $d = 1.188$ for recovery), strawberry ($p = 0.023$, $d = 0.803$and $p = 0.007$, $d = 1.182$, respectively), social 1 ($p < 0.0001$, $d = 1.252$ and $p = 0.001$, $d = 1.184$, respectively) and social 2 ($p < 0.0001$, $d = 1.284$ and $p = 0.004$, $d = 1.024$, respectively) than controls, and that there were no differences between stressed and recovery rats. As expected, when the cumulative time that rats spent sniffing each odor over the 3 trials was analyzed (Fig. 4C), a significant

effect of treatment was found for lemon ($F(2,51) = 11.27$, $p < 0.0001$, $\eta^2_p = 0.306$), strawberry ($F(2,51) = 6.11$, $p = 0.004$, $\eta^2_p = 0.193$), social 1 ($F(2,51) = 11.36$, $p < 0.0001$, $\eta^2_p = 0.308$) and social 2 ($F(2,51) = 9.87$, $p < 0.0001$, $\eta^2_p = 0.279$) odors, but not for water ($F(2,51) = 3.10$, $p = 0.054$, $\eta^2_p = 0.108$). Post-hoc tests showed that stressed and recovery rats spent significantly less time sniffing lemon, strawberry, social 1 and social 2 than control rats, and that there were no significant differences between stressed and recovery rats in any of the odors. When each experimental group was individually analyzed, a significant habituation to each odor was observed in control rats, as shown by the progressive decline in the time spent sniffing water ($F(2,34) = 7.32$, $p = 0.002$, $\eta^2_p = 0.301$), lemon ($F(2,34) = 18.51$, $p < 0.0001$, $\eta^2_p = 0.521$), strawberry ($F(2,34) = 12.68$, $p < 0.0001$, $\eta^2_p = 0.427$), social 1 ($F(2,34) = 14.26$, $p < 0.0001$, $\eta^2_p = 0.456$) and social 2 ($F(2,34) = 14.87$, $p < 0.0001$, $\eta^2_p = 0.467$) odors. Conversely, stressed rats did not habituate to water ($F(2,34) = 2.77$, $p = 0.077$, $\eta^2_p = 0.14$), lemon ($F(2,34) = 0.34$, $p = 0.714$, $\eta^2_p = 0.020$), strawberry ($F(2,34) = 2.41$, $p = 0.105$, $\eta^2_p = 0.124$), social 1 ($F(2,34) = 1.49$, $p = 0.240$, $\eta^2_p = 0.081$) or social 2 ($F(2,34) = 0.06$, $p = 0.947$, $\eta^2_p = 0.003$) odors. Recovery rats behave similarly to controls, as they showed a progressive decline in the time spent sniffing water ($F(2,34) = 9.73$, $p < 0.0001$, $\eta^2_p = 0.364$), lemon ($F(2,34) = 18.33$, $p < 0.0001$, $\eta^2_p = 0.519$), strawberry ($F(2,34) = 7.12$, $p = 0.003$, $\eta^2_p = 0.295$), social 1 ($F(2,34) = 9.84$, $p < 0.0001$, $\eta^2_p = 0.367$) and social 2 ($F(2,34) = 5.95$, $p = 0.006$, $\eta^2_p = 0.259$) odors.

When evaluating cross-habituation (Fig. 4B), we found a significant effect of treatment when a new odor was presented for the first time: water to lemon ($F(2,51) = 11.74$, $p < 0.0001$, $\eta^2_p = 0.315$), lemon to strawberry ($F(2,51) = 7.22$, $p = 0.002$, $\eta^2_p = 0.221$), strawberry to social 1 ($F(2,51) = 10.27$, $p < 0.0001$, $\eta^2_p = 0.287$) and social 1 to social 2 ($F(2,51) = 11.17$, $p < 0.0001$, $\eta^2_p = 0.305$) odors. Post-hoc analysis showed this

effect was driven by both stressed and recovery groups. In fact, stressed as well as recovery rats responded significantly less than controls to the first presentation of lemon ($p < 0.0001$, $d = 1.334$ and $p = 0.001$, $d = 1.087$, respectively), strawberry ($p = 0.006$, $d = 1.128$ and $p = 0.005$, $d = 0.844$, respectively), social 1 ($p = 0.001$, $d = 1.222$ and $p = 0.001$, $d = 0.861$, respectively) and social 2 ($p < 0.0001$, $d = 1.382$ and $p = 0.004$, $d = 0.971$, respectively) odors. No significant differences were found between stressed and recovery rats. When each experimental group was individually analyzed, controls and recovery rats showed significant cross-habituation when presented for the first time to a new odor: water to lemon ($F(1,17) = 24.28$, $p < 0.0001$, $\eta^2_p = 0.588$ and $F(1,17) = 17.39$, $p = 0.001$, $\eta^2_p = 0.506$, respectively), lemon to strawberry ($F(1,17) = 30.24$, $p < 0.000$, $\eta^2_p = 0.640$ and $F(1,17) = 10.07$, $p = 0.006$, $\eta^2_p = 0.383$, respectively), strawberry to social 1 ($F(1,17) = 24.80$, $p < 0.0001$, $\eta^2_p = 0.593$ and $F(1,17) = 19.70$, $p < 0.001$, $\eta^2_p = 0.537$, respectively), and social 1 to social 2 ($F(1,17) = 22.26$, $p < 0.0001$, $\eta^2_p = 0.567$ and $F(1,17) = 5.30$, $p = 0.034$, $\eta^2_p = 0.238$, respectively) odors. Conversely, stressed rats were unable to discriminate between water and lemon ($F(1,17) = 1.83$, $p = 0.194$, $\eta^2_p = 0.097$), lemon and strawberry ($F(1,17) = 3.84$, $p = 0.067$, $\eta^2_p = 0.185$), strawberry and social 1 ($F(1,17) = 2.19$, $p = 0.157$, $\eta^2_p = 0.114$), and social 1 and social 2 ($F(1,17) = 0.38$, $p = 0.546$, $\eta^2_p = 0.022$) odors.

Because both control and recovery groups showed significant cross-habituation (Fig. 4B), but recovery rats explored all odors for a much shorter duration (Fig. 4C), which reflects reduced odor discrimination abilities (Rankin et al., 2009; Wesson et al., 2010) we have calculated the cross-habituation index (Fig. 4D). ANOVA revealed a significant effect of treatment on the cross-habituation index between water and lemon ($F(2,51) = 12.28$, $p < 0.0001$, $\eta^2_p = 0.325$), lemon and strawberry ($F(2,51) = 6.86$, $p = 0.002$, $\eta^2_p = 0.212$), strawberry and social 1 ($F(2,51) = 8.97$, $p = 0.0005$, $\eta^2_p = 0.260$),

and social 1 and social 2 ($F(2,51) = 10.83$, $p = 0.00012$, $\eta^2_p = 0.298$) odors. The cross-habituation index for all odors did not differ between stressed and recovery rats and was, in both groups, significantly smaller than in controls, indicating that after a 4-week stress-free period the discrimination of a new odor from a repeatedly presented habituation odor is still impaired.

3.1.4. CVS impairs, in a partially reversible way, innate attractive and avoidance behaviors

As shown in Figure 5A, treatment significantly influenced the percentage of time spent in the odor corner of the open-field apparatus, compared to water, when rats were exposed to 2PE ($F(2,48) = 5.17$, $p = 0.009$, $\eta^2_p = 0.177$), PEA ($F(2,48) = 8.67$, $p = 0.001$, $\eta^2_p = 0.265$), IPA ($F(2,48) = 8.16$, $p = 0.001$, $\eta^2_p = 0.254$) and CFO ($F(2,48) = 6.31$, $p = 0.004$, $\eta^2_p = 0.208$). Similarly, odors also significantly influenced the percentage of time spent in the odor corner, compared to water, when rats were exposed to 2PE ($F(1,48) = 13.26$, $p = 0.0007$, $\eta^2_p = 0.216$), PEA ($F(1,48) = 9.89$, $p = 0.003$, $\eta^2_p = 0.171$), IPA ($F(1,48) = 17.21$, $p = 0.0001$, $\eta^2_p = 0.264$) and CFO ($F(1,48) = 28.99$, $p < 0.0001$, $\eta^2_p = 0.376$). Moreover, a significant treatment × odor interaction was also found for 2PE ($F(2,48) = 7.98$, $p = 0.001$, $\eta^2_p = 0.249$), PEA ($F(2,48) = 4.71$, $p = 0.0136$, $\eta^2_p = 0.164$), IPA ($F(2,48) = 4.39$, $p = 0.0178$, $\eta^2_p = 0.155$) and CFO ($F(2,48) = 3.80$, $p = 0.030$, $\eta^2_p = 0.137$). Control rats spent significantly more time in the corner containing 2PE and significantly less time in the corner containing PEA, IPA or CFO than in the corner containing water, which indicates that they displayed innate attraction to 2PE and innate aversion to PEA, IPA and CFO. In contrast, stressed rats showed no evidence of attraction to 2PE or avoidance to PEA, IPA and CFO. Recovery rats showed an intermediate, and less uniform pattern of response to the odors tested. They did not

show significant attraction to 2PE, but the time they spent in the corner containing this odor did not significantly differ from that spent by either control or stressed rats. Likewise, they did not show significant aversion to PEA and CFO, but the time they spent in the corner containing PEA was significantly smaller than in stressed rats ($p = 0.005$, $d = 1.388$) and did not differ from that of controls, whereas the time they spent in the corner containing CFO did not differ from that of stressed rats and was significantly greater than in controls ($p = 0.037$, $d = 1.395$). In contrast to the other odors tested, recovery rats showed significant aversion to IPA, but the time they spent in the corner containing the odor did not differ from that of controls and was significantly smaller ($p = 0.003$, $d = 1.480$) than in stressed rats.

In the triple odor test (Fig. 5B), the time spent by rats actively investigating the different corners of the open-field arena was significantly influenced by odors in control rats ($F(3,32) = 67.87$, $p < 0.0001$, $\eta^2_p = 0.864$), but not in stressed ($F(3,32) = 0.059$, $p = 0.980$, $\eta^2_p = 0.006$) and recovery ($F(3,32) = 1.72$, $p = 0.183$, $\eta^2_p = 0.139$) rats. Control rats showed preference to investigate estrus female odors over diestrus female or male odors and water, whereas stressed and recovery rats investigated all odors and water for a similar amount of time.

3.1.5. CVS does not impair male sexual behavior

As shown in Table 1, there was no significant effect of treatment on the latency to first anogenital exploration ($F(2,24) = 1.24$, $p = 0.307$, $\eta^2_p = 0.094$), percentage of cumulative time spent in anogenital exploration ($F(2,24) = 0.10$, $p = 0.903$, $\eta^2_p = 0.008$), latency to mount ($F(2,24) = 0.31$, $p = 0.734$, $\eta^2_p = 0.025$) and mounting frequency ($F(2,24) = 0.20$, $p = 0.821$, $\eta^2_p = 0.016$).

3.1.6. CVS does not impair male-male aggressive behavior

As shown in Table 1, treatment had no effect on the percentage of cumulative time spent in offensive and defensive behaviors ($F(2,48) = 0.27$, $p = 0.763$, $\eta^2_p = 0.011$); all groups spent more time in aggressive than in defensive behaviors ($F(1,48) = 145.76$, $p < 0.0001$, $\eta^2_p = 0.752$), and there was no interaction effect ($F(2,48) = 0.56$, $p = 0.577$, $\eta^2_p = 0.023$). No significant effect of treatment was also found when the cumulative time spent in the different offensive behaviors (Table 1) was analyzed separately: chase ($F(2,24) = 0.79$, $p = 0.466$, $\eta^2_p = 0.062$), attack ($F(2,24) = 0.24$, $p = 0.787$, $\eta^2_p = 0.020$), offensive upright ($F(2,24) = 0.28$, $p = 0.757$, $\eta^2_p = 0.023$), lateral threat ($F(2,24) = 0.15$, $p = 0.861$, $\eta^2_p = 0.012$) and keep down behavior ($F(2,24) = 0.12$, $p = 0.890$, $\eta^2_p = 0.010$).

3.2. Effects of CVS and subsequent recovery on body and organ weights

The effect of treatment on body weight gain (Table 2) was statistically significant ($F(2,51) = 37.73$, $p < 0.0001$, $\eta^2_p = 0.597$). Body weight increase (relative to the body weight at the beginning of CVS exposure or handling) was significantly smaller in stressed than in control and recovery rats. No differences were found between recovery and control rats.

The relative adrenal weight (average adrenal weight in mg/g body weight) was also significantly influenced by treatment ($F(2,51) = 35.20$, $p < 0.0001$, $\eta^2_p = 0.58$). As shown in Table 2, the relative adrenal weight was significantly increased (56%) in stressed relative to control and recovery rats. However, in this group, the relative adrenal weight was still significantly higher (22%) than in control rats.

A significant effect of treatment on relative thymus weight (mg/g body weight) was also revealed by ANOVA ($F(2,51) = 11.53$, $p < 0.0001$, $\eta^2_p = 0.311$). Thymus

weight was significantly smaller (28%) in stressed than in control rats (Table 2). In recovery rats, the weight increased to control values and was, therefore, significantly higher than in stressed rats.

Because there is no direct relationship between variations in body weight and in testis weight (Bailey et al., 2004), we have analyzed the effects of treatment on testicular weight without correction for body weight. Despite the small variations in the average testicular weights, a significant effect of treatment was shown by ANOVA ($F(2,51) = 5.273$, $p = 0.008$, $\eta^2_p = 0.171$). The average testicular weight did not differ between stressed and control rats, but was significantly lower in recovery than in control rats (Table 2).

3.3. Effects of CVS and subsequent recovery on hormone levels

Data are shown in Table 2. Consistent with previous studies that used the same stress paradigm and a similar duration of stress exposure (e.g., Cox et al., 2011; Lu et al., 2015; Pêgo et al., 2008; Ventura-Silva et al., 2013), corticosterone levels measured at the end of CVS exposure were significantly higher in stressed than in control rats ($p < 0.0001$, t-test, $d = 2.249$). At the end of the experiments, there was still a significant influence of treatment on corticosterone concentrations ($F(2,51) = 4.56$, $p = 0.015$, $\eta^2_p = 0.152$). The levels were significantly higher in stressed than in control and recovery rats. No differences were found between recovery and control rats.

Testosterone levels measured at the end of the experiments, were also significantly influenced by treatment ($F(2,51) = 8.89$, $p = 0.001$, $\eta^2_p = 0.259$). The levels were similar in stressed and recovery rats and were, in both groups, significantly lower than in control rats.

3.4. Effects of CVS and subsequent recovery on the nLOT morphology and neurochemistry

As shown in Table 3, treatment did not influence the volume of the nLOT ($F(2,15) = 0.874$, $p = 0.438$, $\eta^2_p = 0.104$) or the volume of its layers 1 ($F(2,15) = 0.73$, $p = 0.499$, $\eta^2_p = 0.088$), 2 ($F(2,15) = 0.47$, $p = 0.634$, $\eta^2_p = 0.059$) and 3 ($F(2,15) = 3.31$, $p = 0.065$, $\eta^2_p = 0.306$). Similarly, the total number of nLOT neurons (Table 3) did not differ among stressed, recovery and control rats in any of the layers (layer 1: $F(2,15) = 0.93$, $p = 0.426$, $\eta^2_p = 0.108$; layer 2: $F(2,15) = 0.84$, $p = 0.453$, $\eta^2_p = 0.10$; layer 3: $F(2,15) = 2.83$, $p = 0.090$, $\eta^2_p = 0.274$) or when the total number of neurons in the nLOT was computed ($F(2,15) = 1.40$, $p = 0.277$, $\eta^2_p = 0.157$).

Conversely, there was a significant effect of treatment in the mean somatic volume of nLOT neurons in layer 2 ($F(2,15) = 89.47$, $p < 0.0001$, $\eta^2_p = 0.923$) as well as in layer 3 ($F(2,15) = 78.70$, $p < 0.0001$, $\eta^2_p = 0.913$). In layer 2, the soma size of neurons was similar in stressed and in recovery rats and was, in both groups, significantly larger than in controls (Fig. 6). In layer 3, neuronal cell bodies were also significantly larger in stressed and recovery rats than in controls. However, the mean soma size was significantly reduced in recovery relative to stressed rats.

As shown in Table 3, treatment did not influence the total number of interneurons expressing the three calcium binding proteins, PV ($F(2,15) = 1.40$, $p = 0.278$, $\eta^2_p = 0.157$), CB ($F(2,15) = 0.96$, $p = 0.406$, $\eta^2_p = 0.113$) and CR ($F(2,15) = 1.17$, $p = 0.338$, $\eta^2_p = 0.135$). According to our results, these interneurons represent about 1.6%, 2.2% and 2.8%, respectively, of the total cell population of the nLOT, showing that, as previously reported (McDonald, 1998; Sah et al., 2003), the nLOT contains a majority of glutamatergic neurons.

Conversely, stress and recovery from stress significantly influenced the mean somatic volume of interneurons expressing PV ($F(2,15) = 5.67$, $p = 0.015$, $\eta^2_p = 0.43$), CB ($F(2,15) = 7.51$, $p = 0.006$, $\eta^2_p = 0.50$) and CR ($F(2,15) = 8.67$, $p = 0.003$, $\eta^2_p = 0.536$). The mean soma size of PV- (Fig. 7), CB- (Fig. 8) and CR-immunoreactive (Fig. 9) neurons was significantly smaller (15%, 31% and 23%, respectively) in stressed than in control rats. After 7 weeks of recovery from stress, the soma size of PV interneurons returned to control values, that of CB interneurons was intermediate between stressed and control rats, but did not significantly differ from both, whereas that of CR interneurons did not differ from that of stressed rats and was, therefore, significantly smaller than in controls.

3.5. Effects of CVS and subsequent recovery on GABA concentrations in the nLOT

As shown in Figure 10, there was a significant effect of treatment on the nLOT GABA content ($F(2,15) = 7.31$, $p = 0.006$, $\eta^2_p = 0.494$). GABA levels did not differ between stressed and recovery rats and were, in both groups, significantly lower than in controls.

4. Discussion

We carried out several standard procedures to examine the effectiveness of the CVS paradigm. Stressed rats had increased plasma corticosterone levels at the end of 4 weeks of CVS exposure as well as after the 3 weeks allowed for behavioral evaluation, indicating that they experienced prolonged corticosterone hypersecretion. Consistent with this, stressed rats also displayed attenuated body weight gain, increased adrenal

weight and decreased thymus weight compared to control rats. Data on anxiety- and depressive-like behaviors were also indicative of chronic hypercorticism. Specifically, stressed rats showed decreased locomotor activity, travelled smaller distances in the outer zone of the open-field and spent more time in the closed arms of the elevated plus-maze than controls, changes that are typically thought to reflect increased anxiety (Prus et al., 2009). Stressed rats also showed decreased sucrose preference, which is interpreted as an index of anhedonia, a core symptom of depression (Muscat and Willner, 1992). Together, these alterations indicate that the CVS paradigm applied was effective. In contrast to stressed rats, recovery rats had corticosterone levels within control levels, their body weight gain and relative thymus weight did not differ from those of controls, and they did not show anhedonia. Yet, their adrenal glands were still heavier than in controls, and data on the parameters used to evaluate anxiety-like behavior were intermediate between, but not significantly from, those of stressed and control rats, indicating that the stress-free period subsequent to CVS attenuated the effects of stress on anxiety.

Particularly interesting, and seldom reported in studies using stress paradigms, was the finding that rats exposed to CVS over 4 weeks display severe olfactory deficits with inability to detect and discriminate between odors. Stressed rats spent significantly more time to find the hidden cookie than control rats, a difference that is not likely to be due to motivational, motor or sensory deficiencies because they performed as well as controls in the surface cookie test. Moreover, in the olfactory habituation/cross-habituation test, stressed rats did not display decreasing durations of investigative behavior when repeatedly exposed to the same odor (habituation) or increased investigative behavior when a new odor was presented (cross-habituation), showing that they have decreased olfactory sensitivity. Notably, recovery rats spent the same time as

stressed rats, and thus significantly more time than controls, to find the hidden cookie, suggesting that the olfactory deficits provoked by CVS are long-lasting. It was, therefore, unexpected to find that recovery rats displayed habituation to all odors tested and cross-habituation when a new odor was presented, despite the overall reduction in the time they spent investigating the habituation odors and the new odor. However, the analysis of the cross-habituation index revealed that this parameter does not differ between recovery and stressed rats and is, in both groups, significantly smaller than in controls, showing that, more than 4 weeks after the end of CVS exposure, rats still have poor odor discrimination abilities (Wesson et al., 2010). Altogether, these findings indicate that CVS severely disrupts olfactory functions and that this effect is not modified appreciably for more than 4 weeks after the last stress exposure.

From all cortical centers in the brain that receive projections from the main olfactory bulb, only the anterior olfactory nucleus and the cortical amygdala receive topographically organized projections, suggesting that they are involved in generating specialized functions (Miyamichi et al., 2011; Sosulski et al., 2011). In particular, the posterolateral cortical nucleus seems to be essential for mediating approach and avoidance behaviors towards attractive and aversive odors (Root et al., 2014), and the same is true for the nLOT, as recently demonstrated after bilateral excitototoxic lesions of the nucleus (Vaz et al., 2017). We therefore extended our study to examine if CVS disrupts the innate capacity of rats to differentiate between attractive and aversive odors. Contrary to controls, stressed rats showed no attraction to 2PE or avoidance towards PEA, IPA and CFO. Recovery rats showed a trend towards attraction to 2PE and avoidance to PEA, but the differences to stressed rats were only statistically significant for PEA. Moreover, like stressed rats, recovery rats did not show avoidance to CFO, but, contrary to stressed rats, they responded with avoidance behavior to IPA. With the

exception of CFO, which is a vomeronasal organ-activating odor, all odors tested are detected by the main olfactory epithelium. These different odor classes are known to activate distinct neural pathways that include, respectively, several limbic system nuclei and the medial defensive hypothalamic system (reviewed in Canteras et al, 2015; Gross and Canteras, 2012; Li and Liberles, 2015). Thus, present data suggest that the receptors and/or the neural centers of the main and accessory olfactory systems might differ with respect to their vulnerability to chronic stress, with its effects being transient, or at least partially reversible, in the main olfactory system after a 6 week-stress free period.

To further characterize the effects of CVS on the main olfactory system, we next examined if CVS interferes with the recognized attraction that sexually-inexperienced male rats have to volatile olfactory cues from estrus females when compared to diestrus females or males (reviewed in Petrulis, 2013). Our data showed that contrary to controls, who spent about 40% more time investigating the odor stimulus of estrus over diestrus or male rats, stressed rats had no preference for estrus female odors, an effect that was still not overcome after a 6-week stress-free period. These findings are not necessarily at odds with the suggested transient effects of stress on the main olfactory system because recovery, as well as stressed, rats have decreased testosterone levels and it is known that male rodent's interest in female odors is modulated by activational effects of testosterone (reviewed in Petrulis, 2013). Altogether, these experiments reveal that CVS severely impairs specialized chemosensory behavior, irrespective of the receptor or olfactory system activated by the odors, and that after a 6-7 week stress-free period these behaviors are only partially restored.

Because there is ample evidence that the main and accessory olfactory systems interact to promote male-specific behaviors (Hashikawa et al., 2016), we next examined if CVS exposure and subsequent recovery interfere with male sexual behavior and

aggression. We found that stressed and recovery rats displayed the same interest as controls towards sexually receptive females when they were allowed to interact physically with them. Also, stressed and recovery rats did not differ from controls in the latency to first anogenital exploration and mounting and duration of anogenital exploration, and consistently mounted sexually-receptive females, not differing in the mounting frequency. In the resident-intruder test, stressed and recovery rats performed similarly to controls, spending more time in offensive behaviors towards the intruders than in defensive behaviors. In this regard, it is noteworthy that stressed and recovery rats displayed undisturbed sexual and aggressive behaviors despite having low testosterone levels, indicating, as previously reported (Damassa et al., 1977; Wood et al., 2013), that in sexually active adult male rats there is no direct correlation between plasma testosterone levels and any measure of sexual or aggressive behavior. Earlier studies have shown that stressed rats, when tested during CVS exposure, have reduced sexual activity and aggressive behaviors, and increased defensive behaviors (D'Aquila et al., 1994; Grønli et al., 2005; Wang et al., 2012). In view of these data, our results indicate that the effects of CVS on sexual and aggressive behaviors are short-lived and fade away quickly after the end of stress exposure. They also suggest that after CVS exposure the activity of the accessory olfactory system is promptly re-established if this system is activated by social investigation.

It was shown that CVS, in addition to decreasing responses, measured by electro-olfactograms, to odorants, increases neuronal apoptosis in olfactory mucosa (Raynaud et al., 2015). Data obtained in this study extends these previous findings by demonstrating that a neural center that receives direct projections from the olfactory bulb, and is therefore regarded as a component of the olfactory system, the nLOT, also displays structural and neurochemical changes is response to CVS. It was also reported

that a 10-day exposure to CVS leads to dendritic hypertrophy in the basolateral amygdala (Vyas et al., 2002), whereas a 4-week CVS exposure does not alter the morphology of the basolateral, basomedial, lateral and central amygdalar nuclei (Pêgo et al, 2008). On the basis of these data, the results we have obtained in the nLOT seem to indicate that the cortical amygdala, from which the nLOT is also considered as an integral part, is more vulnerable to CVS than the remaining amygdalar nuclei. Specifically, we found that CVS and subsequent recovery do not alter the volume of the nLOT or the total number of its neurons and interneurons producing PV, CB and CR. However, our data shows that CVS induces somatic hypertrophy of nLOT neurons and atrophy of PV, CB and CR interneurons, indicating that only the output neurons of the nLOT, which are glutamatergic, are hypertrophied in stressed rats. Atrophy of nLOT interneurons was accompanied by a significant reduction in local GABA levels, suggesting that the hypertrophy, and possibly hyperactivity, of nLOT projecting neurons, is consequent to impaired inhibition due to effects of high glucocorticoid levels on the activity of GABA interneurons, as already observed in the basolateral amygdala (reviewed in Radley et al., 2015). These effects seem to be irreversible or at least long-lasting because, after a 7-week stress-free period, nLOT neurons remained hypertrophied, CB- and CR-producing interneurons were still atrophied, with only PV interneurons having regained their normal volume, and the nLOT levels of GABA were still significantly reduced relative to those of controls.

The reasons why structural alterations of the nLOT influence olfactory-guided behaviors are yet unexplored. However, and taking into account the bi-directional connections established by the nLOT, and particularly by its layer 2, with the olfactory bulb, anterior olfactory nucleus and piriform cortex (Price, 1973; Luskin and Price, 1983; Santiago and Shammah-Lagnado, 2004), our data allow us to hypothesize that the

CVS-induced changes that we have observed in the nLOT might influence olfactory sensitivity and odor discrimination as these functions have been classically ascribed to the aforementioned areas. Future studies addressing the specific influence of the nLOT in these particular areas, could shed light on our knowledge of the role played by this nucleus in olfactory-guided behaviors.

5. Conclusion

Data obtained in the present study shows that CVS severely impairs odor detection and discrimination, and disrupts attraction and aversion behaviors to volatile odors. They also show that these effects are long-lasting, as they persist for more than 4 weeks after the last stress exposure. These functional and behavioral effects are associated with long-lasting, potentially irreversible, structural and neurochemical alterations of the nLOT, a component of the cortical amygdala where convergent olfactory and vomeronasal information is likely to be processed.

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Figure legends

Fig. 1

Experimental timeline of the stress paradigm protocol and sequence of behavioral tests. The beginning of chronic variable stress (CVS) was used as day 0 (E0) of the experiments. After day 28 (E28), rats of the recovery group were left undisturbed for an additional 28 days (Recovery). Rats of the stressed group started to be exposed to CVS at E28. Control rats were handled during the total experimental period (E0-E56). The end of the experimental period (E56) corresponds to day 0 (B0) of behavioral testing. B3-B18, indicate the days at which the different behavioral tests were performed and B21, day of sacrifice.

Fig. 2

Effect of CVS and subsequent recovery on anxiety-like behaviors. (A) Open-field test. The histogram shows the mean + SEM distances travelled in outer and inner zones of the open-field. (B-C), Elevated plus-maze test. Graphic representation of the mean + SEM time spent (B) and distances travelled (C) in the closed and open arms, and in the central square of the elevated plus-maze. (D) The histogram shows the mean + SEM number of fecal boli and amount of urine deposited by rats during the open-field and elevated plus-maze tests. *$p < 0.05$, **$p < 0.001$ compared to controls; $^{\#}p < 0.001$ compared with the inner zone in the open-field test and with the open arms and central square in the elevated plus-maze.

Fig. 3

Effect of CVS and subsequent recovery on sucrose preference. The histogram shows the mean + SEM volume of sucrose solution and water ingested by each group of rats in the sucrose preference test averaged over the 4 days of the test. *$p < 0.001$ compared to controls; $^{+}p < 0.01$ compared to recovery rats; $^{\#}p < 0.001$ compared with sucrose intake of the respective group.

Fig. 4

Effect of CVS and subsequent recovery on olfactory functions. (A) Buried food test. Histograms show the mean + SEM time spent to find the hidden and the surface cookies. (B-D) Olfactory habituation/cross-habituation test. The graph (B) shows mean ± SEM olfactory investigation times, across 3 consecutive 2-min trials separated by 1-min inter-trial intervals, of water, 2 nonsocial and 2 social odors. The values of the post-hoc tests are shown in the Results section. The histogram (C) shows the mean + SEM cumulative time that rats spent investigating each odor. The graph (D) shows mean + SEM cross-habituation indices between odors. W, water; L, lemon; St, strawberry; S1, social 1 odor; S2, social 2 odor. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to controls; $^{\#}p < 0.001$ compared to the respective group in the buried cookie test.

Fig. 5

Effect of CVS and subsequent recovery on innate attractive or aversive behaviors. (A) The histogram shows the mean + SEM percentage of cumulative time over the duration of the test that rats spent investigating the corner of the open-field arena where the several odors were individually presented. The percentage of time spent investigating water (dashed line) was used as the criterion to define the threshold between attraction

and aversion. 2PE, 2-phenylethanol; PEA, 2-phenylethylamine; IPA, isopentylamine; CFO, cat fur odor. (B) Graphic representation of the mean + SEM percentage of cumulative time over the duration of the test that rats spent investigating the corners of the open-field arena where the Petri dishes containing the odors of receptive females, non-receptive females, males and water (one in each corner) were located. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to water of the respective experimental group; $^{\#}p < 0.001$ compared to other odors and water.

Fig. 6

Digital photomicrographs of Giemsa-stained glycolmethacrylate-embedded coronal sections through the nLOT and graphic representation of the estimates of mean somatic volumes of neurons in nLOT layers 2 (nLOT2) and 3 (nLOT3). (A) Section through the nLOT of a control rat, where the layers and the cytoarchitectonic features of the nLOT can be seen. The continuous line delineates the nLOT and the dashed lines mark the borders between adjacent layers. Briefly, layer 1 lies next to the pia and contains very few neurons; layer 2 is disc-shaped, lies dorsally to layer 1 and is densely populated with cells; layer III has a triangular shape and lies dorsally to layer 2. (B) Graphic representation of the effects of treatment on the mean somatic volume of neurons in layers 2 and 3 of the nLOT. Columns represent means and vertical bars + SD. (C-H) Photomicrographs showing representative neurons of layer 2 (C-E) and layer 3 (F-H) of control (C, F), stressed (D, G) and recovery (E, H) rats. Layer 2 is densely populated, and is defined by its homogeneous pyramidal cells. Layer 3 is characterized by its cell polymorphism and by the presence of the largest pyramidal cells of the nLOT. In layer 2, the somatic volumes are larger in stressed (D) and recovery (E) rats than in the control rat (C). The volumes do not differ between stressed and recovery rats. In layer 3,

the larger size of the neuronal cell bodies of the stressed rat (G) relative to those of control (F) and recovery (H) rats is evident. The somatic volumes are also larger in recovery (H) than in control (F) rats. *$p < 0.001$, compared to the respective controls; $^{+}p < 0.001$, compared to recovery rats. Scale bars 200 μm in A; 40 μm in C-H.

Fig. 7

Digital photomicrographs of coronal sections through the nLOT immunostained for parvalbumin (PV) and graphic representation of the estimates of mean somatic volumes of PV-immunoreactive neurons. (A) Representative section through the nLOT of a control rat. Most of the PV-immunoreactive neurons are located in layers 2 and 3. Neurons of layer 2 have dendrites that extend to layer 1. The density of PV-immunoreactive terminals is high in layers 2 and 3. (B) Graphic representation of the effects of treatment on the mean somatic volume of PV-immunoreactive neurons. Columns represent means and vertical bars + SD. (C-E) High magnification of PV-immunoreactive neurons from a control (C), a stressed (D) and a recovery (E) rat. The smaller size of the neuronal cell bodies in the stressed rat (D) relative to control (C) and recovery (E) rats is evident. Neuronal volumes do not differ between control (C) and recovery (E) rats. *$p < 0.05$, compared with control rats; $^{+}p < 0.05$, compared with recovery rats. Scale bars 200 μm in A; 40 μm in C-E.

Fig. 8

Digital photomicrographs of coronal sections through the nLOT immunostained for calbindin (CB) and graphic representation of the estimates of mean somatic volumes of CB-immunoreactive neurons. (A) Representative section through the nLOT of a control rat. The majority of the neurons that contain CB are located in layer 3. The density of

the labeling of the neuropil is high throughout the nLOT, with exception of the outer half of the layer 1, which is lightly stained. (B) Graphic representation of the effects of treatment on the mean somatic volumes of CB-immunoreactive neurons. Columns represent means and vertical bars + 1 SD. (C-E) High magnification of nLOT neurons immunoreactive for CB from a control (C), a stressed (D) and a recovery (E) rat. The smaller size of the neuronal cell bodies in the stressed rat (D) relative to the control rat (C) is evident. No significant differences were found between the mean somatic volumes of recovery rats (E) and those of control (C) and stressed (D) rats. *$p < 0.01$, compared with control rats. Scale bars 200 μm in A; 40 μm in C-E.

Fig. 9

Digital photomicrographs of coronal sections through the nLOT immunostained for calretinin (CR) and graphic representation of the estimates of mean somatic volumes of CR-positive neurons. (A) Representative section through the nLOT of a control rat. Most CR-positive neurons are located in the layer 2. A few neurons can be seen scattered throughout layer 3 and the deep part of layer 1. The labeling of the neuropil is very low, which contrasts with the intense staining of the adjacent areas. The superficial third of layer 1 contains a zone of intensely staining CR-positive neuropil, whereas in the inner third of the same layer thick bundles of CR-immunoreactive fibers can be seen. (B) Graphic representation of the effects of treatment on the mean somatic volume of CR-immunoreactive neurons. Columns represent means and vertical bars + SD. (C-E) High magnification of CR-immunoreactive neurons from a control (C), a stressed (D) and a recovery (E) rat. The smaller size of the neuronal cell bodies in the stressed (D) and recovery (E) rats relative to the control rat (C) is easily recognizable. No

differences were found in the somatic volumes between stressed (D) and recovery (E) rats. *p < 0.01, compared with control rats. Scale bars 200 μm in A; 40 μm in C-E.

Fig. 10

Effects of CVS and subsequent recovery on GABA levels in the nLOT. The histogram shows the GABA concentrations in the nLOT. *p < 0.05 and **p < 0.01 compared to controls.

Table 1. Male sexual and aggressive behavior of control, stressed and recovery rats

	Control	Stress	Recovery
Latency to anogenital exploration (s)	2.33 ± 0.29	3 ± 0.37	2.44 ± 0.29
Time spent in anogenital exploration (s)	39.04 ± 4.45	38.87 ± 2.29	41.11 ± 4.51
Latency to mount (s)	37 ± 5.96	40.78 ± 10.09	31 ± 9.76
Number of mounts per minute	1.27 ± 0.23	1.52 ± 0.31	1.39 ± 0.29
Cumulative time (%)			
Offensive behaviors	15.75 ± 2.05	18.19 ± 2.80	15.95 ± 1.44
Defensive behaviors	1.72 ± 0.49*	0.96 ± 0.40*	1.00 ± 0.38*
Time spent in offensive behaviors (s)			
Chase	2.85 ± 0.56	3.81 ± 0.55	3.59 ± 0.58
Attack	2.86 ± 0.57	3.03 ± 0.40	2.61 ± 0.24
Offensive upright	2.33 ± 0.33	2.75 ± 0.52	2.47 ± 0.33
Lateral threat	3.42 ± 0.92	3.81 ± 0.96	3.14 ± 0.70
Keep down	4.28 ± 1.20	4.79 ± 1.04	4.14 ± 0.67

Data are presented as mean ± SEM. *$p < 0.001$ compared to offensive behaviors of the respective experimental group.

Table 2. Body weight gain, organ weights and hormone levels

	Control	Stress	Recovery
Body weight gain (g)	76 ± 3.62	36 ± 2.62[a]	79 ± 5.06
Adrenal weight (mg/g)	0.060 ± 0.002	0.094 ± 0.003[a]	0.073 ± 0.003[b]
Thymus weight (mg/g)	1.15 ± 0.04	0.83 ± 0.04[c,d]	1.08 ± 0.07
Testis weight (g)	2.95 ± 0.05	2.80 ± 0.04	2.70 ± 0.07[e]
Corticosterone (ng/ml)			
End of stress protocol	69.2 ± 3.13	109.1 ± 5.06[f]	-----
End of experiment	72.1 ± 3.84	100.8 ± 12.77[g,h]	70.3 ± 3.87
Testosterone (ng/ml)			
End of experiment	0.950 ± 0.097	0.494 ± 0.108[i]	0.486 ± 0.052[j]

Data are presented as mean ± SEM. [a]$p < 0.0001$ when compared to control and recovery rats; [b]$p = 0.0061$ when compared to control rats; [c]$p < 0.0001$ when compared to control rats; [d]$p = 0.0026$ when compared to recovery rats; [e]$p = 0.0060$ when compared to control rats; [f]$p < 0.0001$ when compared to control rats; [g]$p = 0.0378$ when compared to control rats; [h]$p = 0.0256$ when compared to recovery rats; [i]$p = 0.0019$ when compared to control rats; [j]$p = 0.0016$ when compared to control rats

Table 3. Stereological data obtained in the nLOT of control, stressed and recovery rats

		Control	Stress	Recovery
Volume (mm^3)	Layer 1	0.097 ± 0.006	0.097 ± 0.010	0.092 ± 0.006
	Layer 2	0.090 ± 0.008	0.093 ± 0.007	0.090 ± 0.004
	Layer 3	0.044 ± 0.005	0.050 ± 0.005	0.048 ± 0.003
	nLOT	0.231 ± 0.015	0.240 ± 0.017	0.231 ± 0.012
Total Neuron Numbers	Layer 1	948 ± 93	979 ± 68	1030 ± 146
	Layer 2	15740 ± 794	15747 ± 345	15304 ± 797
	Layer 3	2370 ± 144	2715 ± 304	2572 ± 279
	nLOT	19057 ± 610	19441 ± 447	18906 ± 636
Total Number of Interneurons	Parvalbumin	285 ± 31	327 ± 59	328 ± 58
	Calbindin	462 ± 104	427 ± 61	390 ± 98
	Calretinin	528 ± 31	496 ± 77	555 ± 82

Data are presented as mean ± SD.

Fig. 1

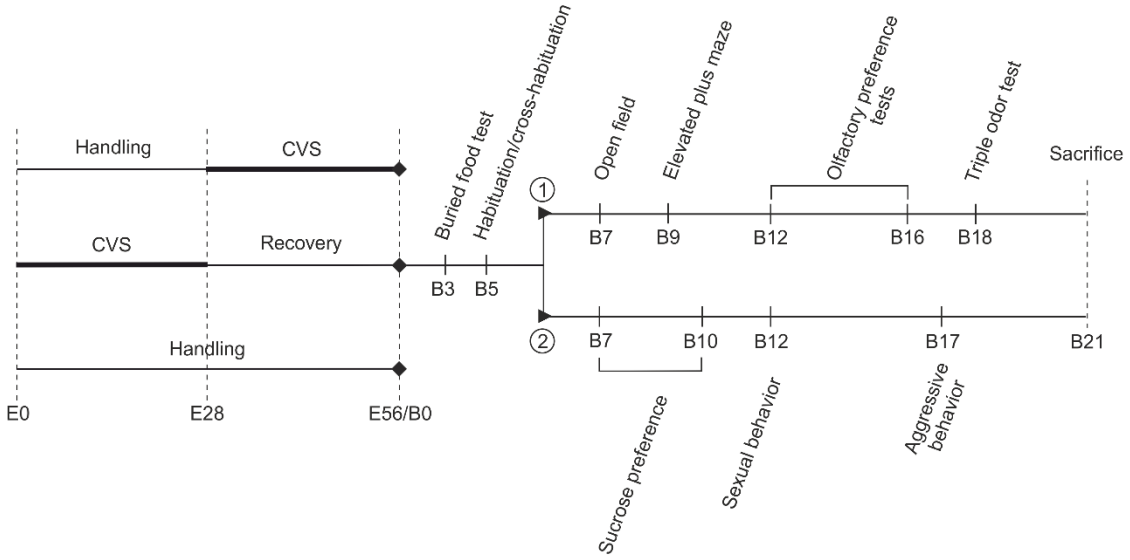

Handling
CVS
CVS
Recovery
Handling
E0
E28
E56/B0
B3
B5
Buried food test
Habituation/cross-habituation
1
2
B7
B9
B12
B16
B18
Open field
Elevated plus maze
Olfactory preference tests
Triple odor test
Sacrifice
B7
B10
B12
B17
B21
Sucrose preference
Sexual behavior
Aggressive behavior

Fig. 2

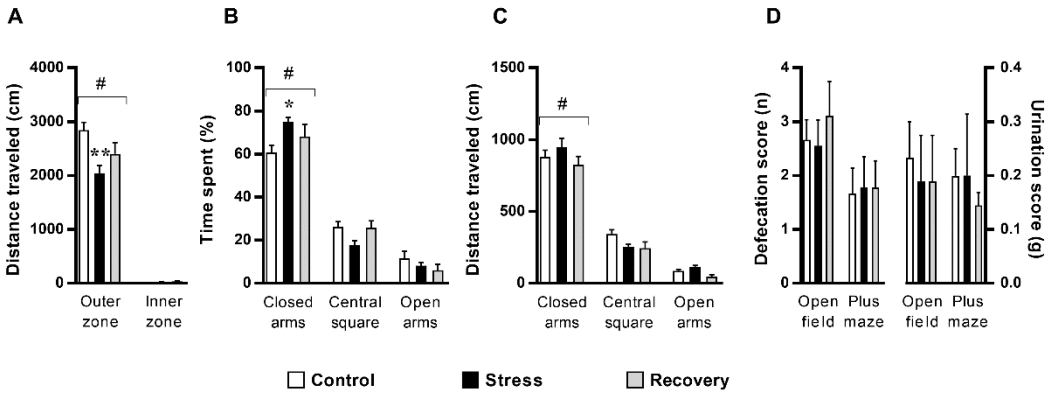

A
B
C
D
Distance traveled (cm)
Time spent (%)
Defecation score (n)
Urination score (g)
Outer zone
Inner zone
Closed arms
Central square
Open arms
Open field
Plus maze
Control
Stress
Recovery

Fig. 3

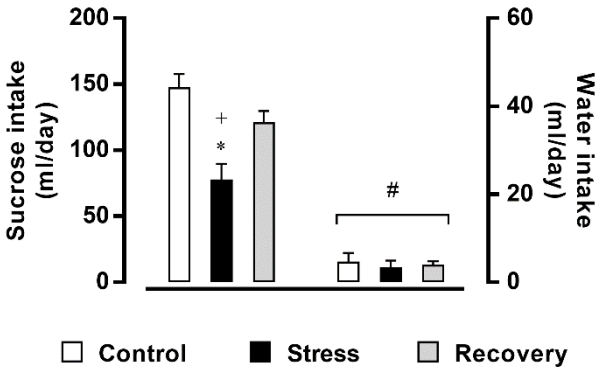

Sucrose intake (ml/day)
200
150
100
50
0
60
40
20
0
Water intake (ml/day)
+
*
#
Control
Stress
Recovery

Fig. 4

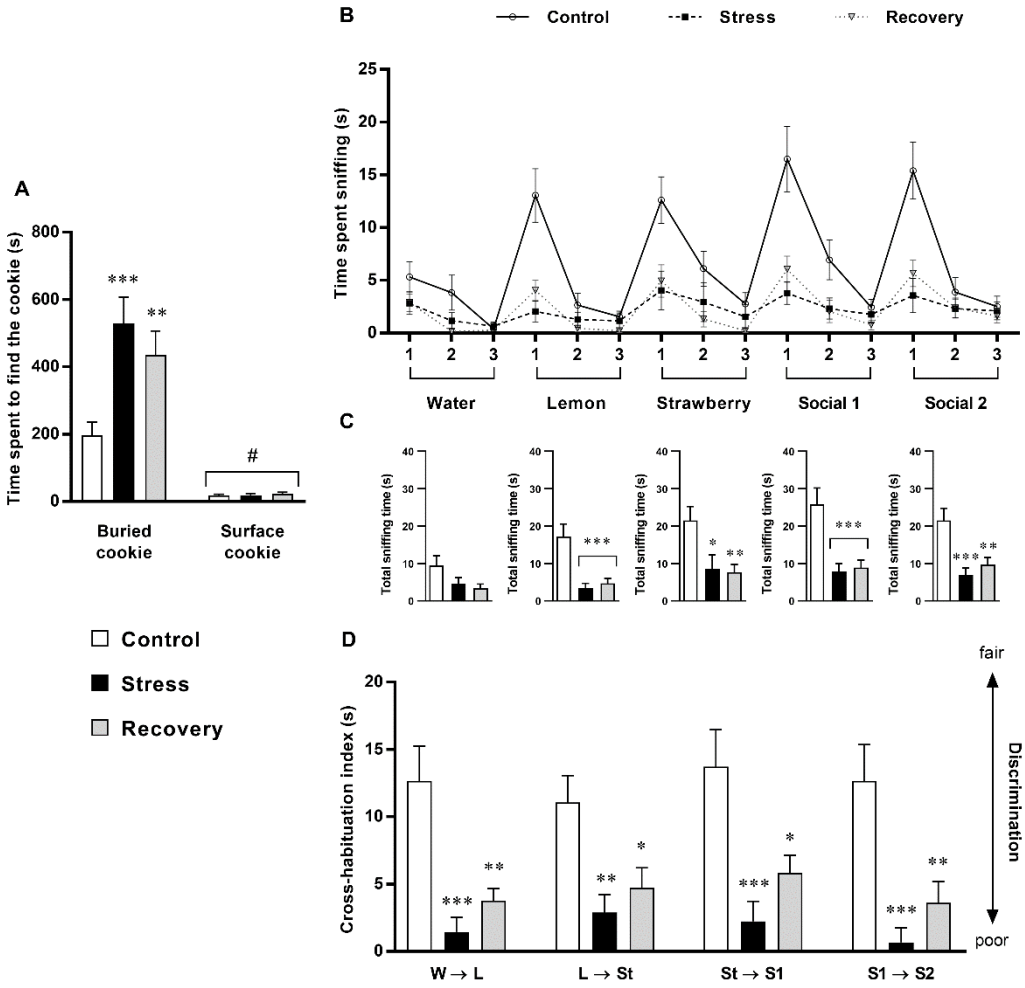

A
Time spent to find the cookie (s)
800
600
400
200
0
***
**
#
Buried cookie
Surface cookie
Control
Stress
Recovery
B
Control
Stress
Recovery
Time spent sniffing (s)
25
20
15
10
5
0
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Water
Lemon
Strawberry
Social 1
Social 2
C
Total sniffing time (s)
40
30
20
10
0
***
*
**
***
***
**
D
Cross-habituation index (s)
20
15
10
5
0
***
**
**
***
*
***
*
**
fair
poor
Discrimination
W → L
L → St
St → S1
S1 → S2

Fig. 5

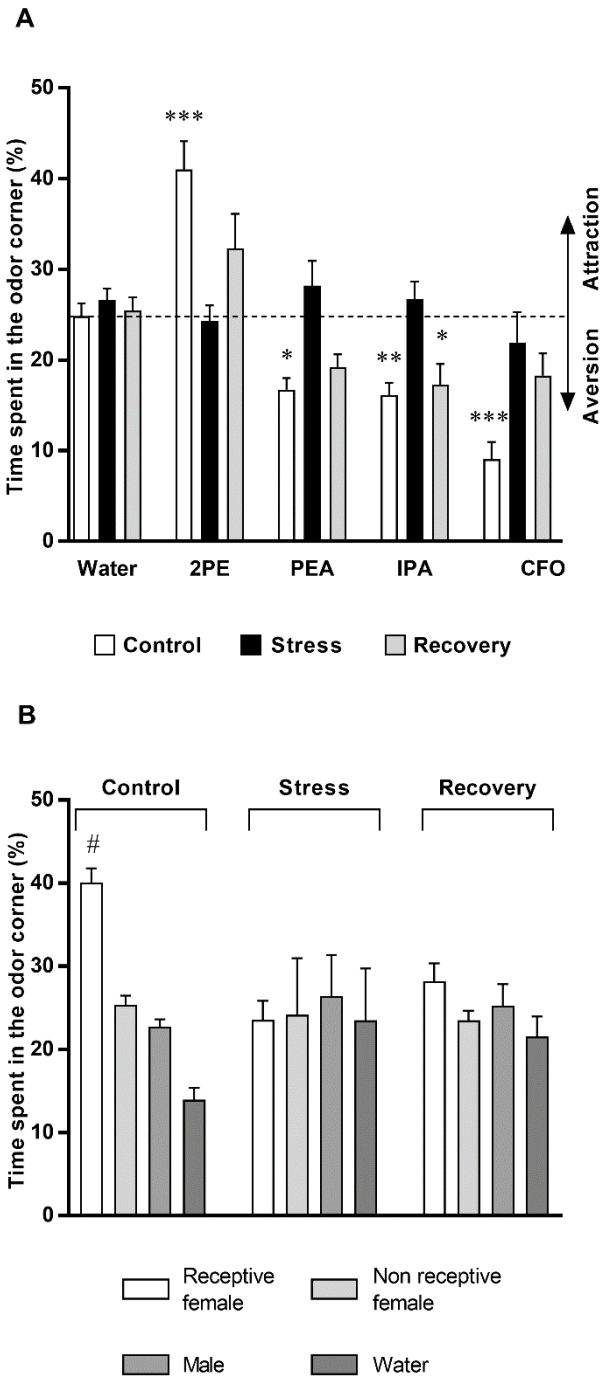

A
Time spent in the odor corner (%)
50
40
30
20
10
0
***
*
**
*
***
Attraction
Aversion
Water
2PE
PEA
IPA
CFO
Control
Stress
Recovery
B
Control
Stress
Recovery
#
Receptive female
Non receptive female
Male
Water

Fig. 6

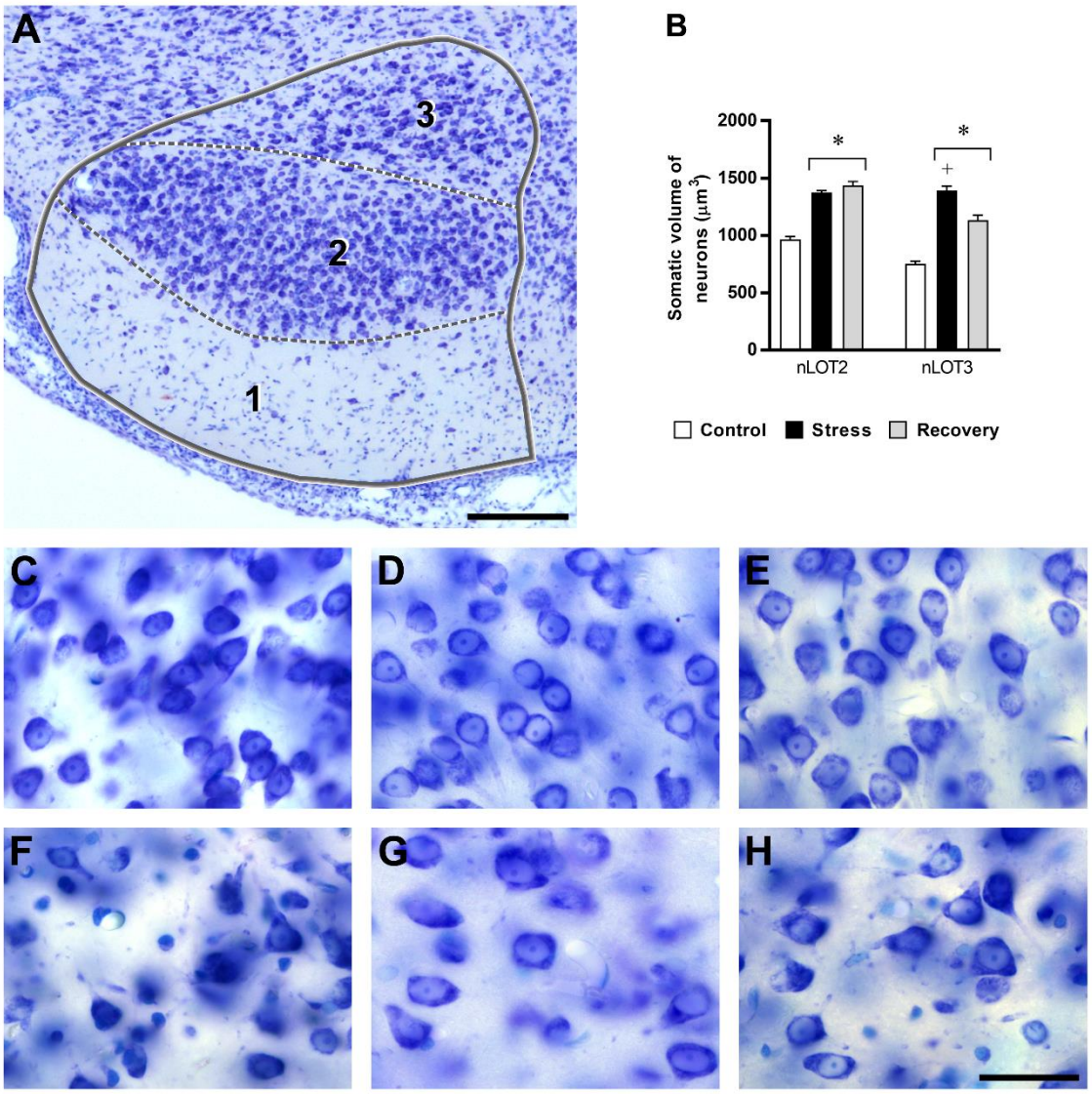

A
3
2
1
B
Somatic volume of neurons (μm³)
2000
1500
1000
500
0
*
*
+
nLOT2
nLOT3
Control
Stress
Recovery
C
D
E
F
G
H

Fig. 7

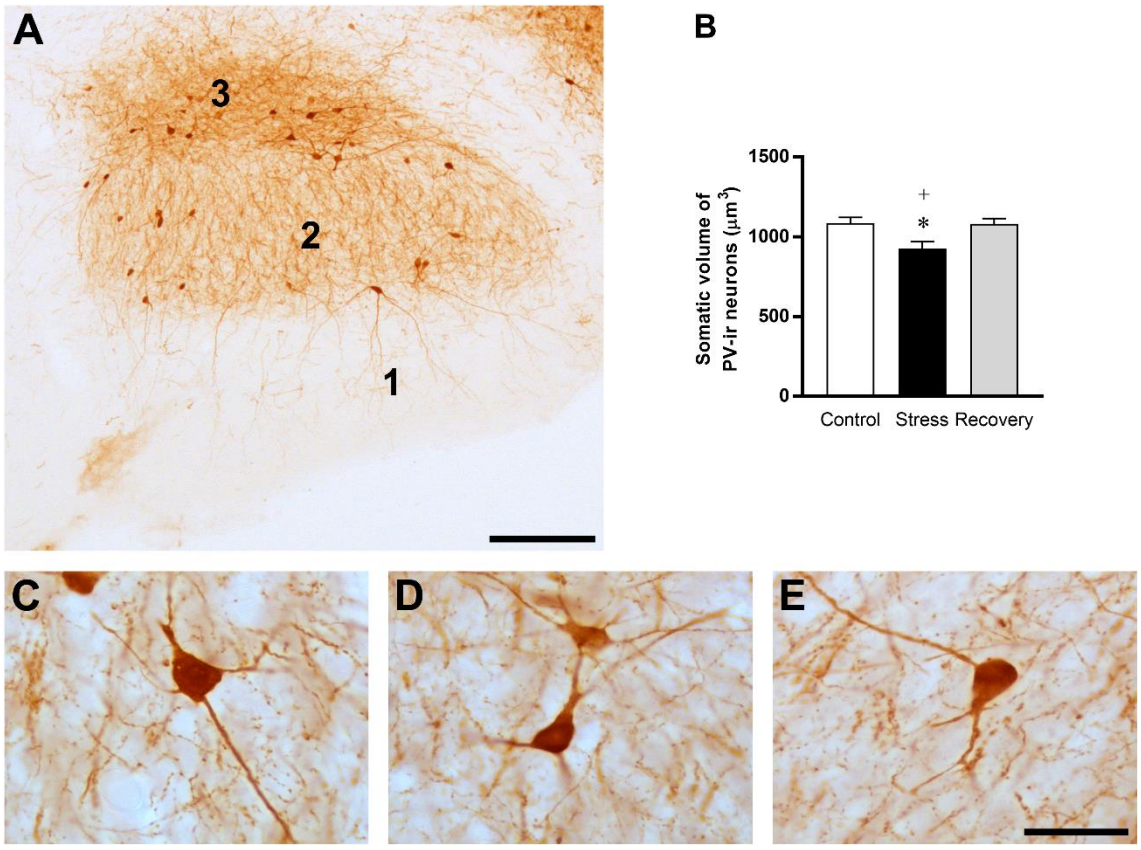

A
3
2
1
B
1500
1000
500
0
Somatic volume of PV-ir neurons (μm³)
+
*
Control
Stress
Recovery
C
D
E

Fig. 8

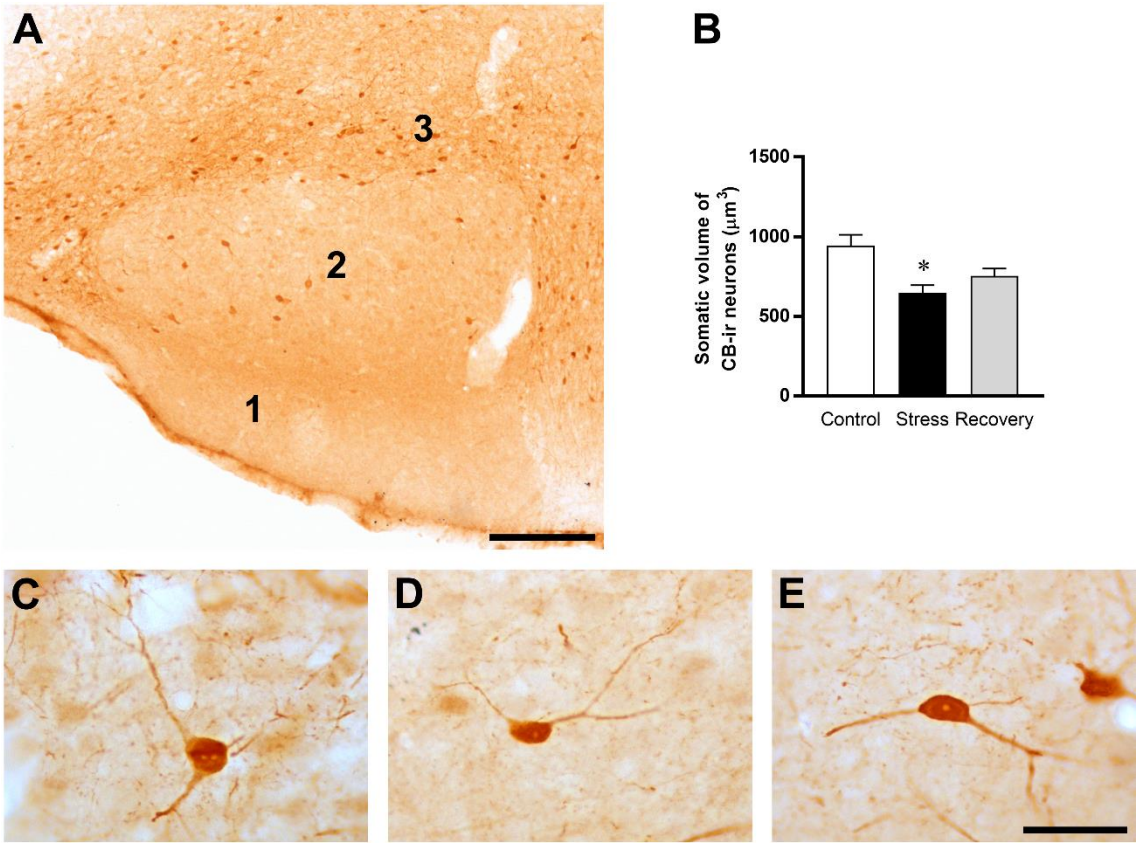

A
3
2
1
B
Somatic volume of CB-ir neurons (μm³)
1500
1000
500
0
*
Control
Stress
Recovery
C
D
E

Fig. 9

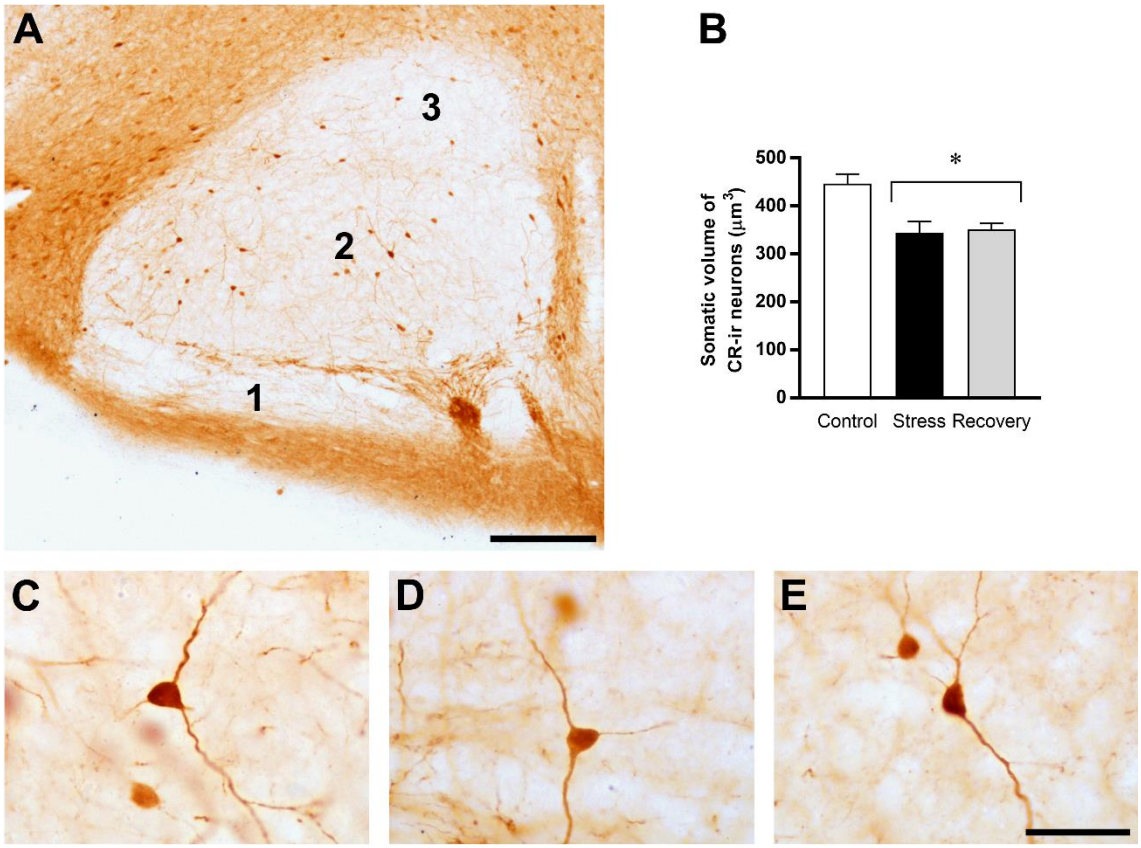
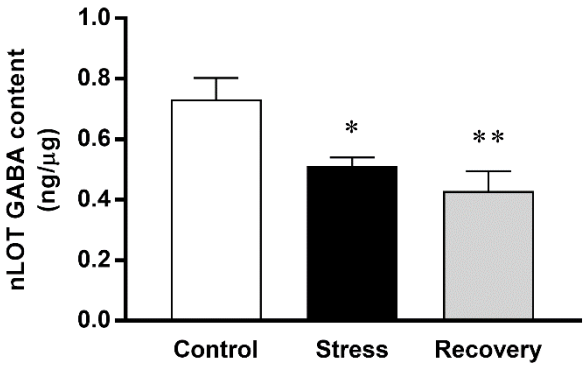

A
3
2
1
B
Somatic volume of CR-ir neurons (μm³)
500
400
300
200
100
0
*
Control
Stress
Recovery
C
D
E

Fig. 10


nLOT GABA content
(ng/μg)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Control
Stress
Recovery

DISCUSSÃO GERAL

Os estudos que constam do TRABALHO 1 desta dissertação permitiram caracterizar os parâmetros morfométricos básicos do nLOT. Verificámos que o nLOT tem um volume aproximado de 0.24 mm^3 e contém cerca de 19,000 neurónios. A maioria (82%) destes neurónios está localizada na camada 2, estando os restantes distribuídos pelas camadas 1 (5%) e 3 (13%). Estas diferenças numéricas têm repercussão na densidade celular, que é particularmente elevada na camada 2, intermédia na camada 3 e muito baixa camada 1, compreendendo-se, assim, que a sua organização tri-laminar seja muito facilmente observável. Em sintonia com os dados obtidos por Millhouse e Uemura-Sumi (1985) em material impregnado pelo método de Golgi, verificámos que os neurónios da camada 2 possuem, em média, corpos celulares de maior volume do que os da camada 3. Estes dados mostram que a variabilidade do volume dos corpos celulares dos neurónios da camada 3 é elevada, já que se sabe ser nesta camada que se encontram localizados as células de maiores dimensões do nLOT (Millhouse e Uemura-Sumi, 1985).

Os dados obtidos nos TRABALHOS 1 e 3 desta dissertação, permitiram ainda quantificar as populações GABAérgicas do nLOT. Verificámos que os neurónios imunorreactivos para a PV, CB, CR, VIP e NPY correspondem a aproximadamente 1,6%, 2,2%, 2,8%, 0,6% e 1,6%, respectivamente, da população neuronal total do núcleo, o que indica que no nLOT o número de neurónios glutamatérgicos excede largamente o dos neurónios GABAérgicos. Do ponto de vista genérico, estes dados estão de acordo com observações, ainda que de carácter não quantitativo, realizadas anteriormente por outros autores (McDonald, 1983), que sugeriram existir grande semelhança entre o nLOT e a divisão basolateral da amígdala. É, no entanto, de salientar que, do ponto de vista quantitativo, as semelhanças não são absolutas já que naquela divisão da amígdala predominam os neurónios imunorreactivos para a CB, sendo os imunorreactivos para a CR apenas cerca de 40% daqueles (McDonald e Mascagni, 2002). Tal não se verifica no nLOT onde as nossas estimativas demonstram que os neurónios imunorreactivos para a CR excedem numericamente os imunorreactivos para a CB.

Tendo em vista a identificação das atribuições funcionais do nLOT, procedemos à realização de lesões selectivas dos neurónios do nLOT através da injecção estereotáxica de ácido quinolínico. Este ácido é um metabolito do triptofano que está presente em concentrações nano-molares no cérebro humano e no líquido cefalorraquidiano (para

revisão, ver Lugo-Huitrón et al., 2013). Quando presente em elevadas concentrações, produz excitotoxicidade por activação dos receptores NMDA (N-metil-D-aspartato) do glutamato. A injecção intracerebral de ácido quinolínico provoca marcada perda neuronal na área injectada sem, no entanto, lesar os axónios de passagem e os terminais nervosos de origem extrínseca. Os estudos comportamentais foram realizados apenas nos ratos em que a extensão da lesão ocupava, no mínimo, 50% do volume das camadas celulares, sem que houvesse lesão significativa das áreas adjacentes. Os resultados destes estudos constam do TRABALHO 2 da presente dissertação.

Verificámos que as lesões do nLOT provocam alterações do peso corporal, quantidade de tecido adiposo e níveis de leptina em tudo idênticas às observadas nos ratos do grupo *sham*, o que sugere que as alterações observadas nestes parâmetros após lesão do nLOT são apenas consequentes ao procedimento estereotáxico. Estas observações sugerem que o nLOT não desempenha papel importante na regulação da ingestão alimentar ou da homeostasia energética, ao contrário do sugerido por outros autores com base nas projecções deste núcleo para áreas cerebrais envolvidas no comportamento alimentar (Santiago e Shammah-Lagnado, 2004).

Os ratos lesados também não apresentavam alterações da actividade locomotora ou comportamentos sugestivos de depressão, aumento da ansiedade ou medo. A verificação da normalidade destes comportamentos foi algo surpreendente, dada a existência de conexões entre o nLOT e a divisão basolateral da amígdala, e de projecções do nLOT para o córtex pré-frontal. Sabe-se que estas regiões estão envolvidas na modulação das respostas a estímulos com conteúdo emocional, tendo sido descrito, tanto no Homem como em modelos animais, que a amígdala, e particularmente a sua divisão basolateral, está envolvida nas respostas emocionais condicionadas por eventos aversivos (para revisão, ver Rosen, 2004; Gross e Canteras, 2012), e que tanto a amígdala como o córtex pré-frontal participam na expressão de comportamentos indiciadores de ansiedade (para revisão, ver Davidson, 2002; Canteras et al., 2010).

Foi com surpresa que verificámos que as lesões do nLOT provocam anosmia e eliminam a capacidade de discriminação de odores. De facto, embora o nLOT receba aferências provenientes do bolbo olfactivo, o que levou Swanson e Petrovich (1998) a considerá-lo um componente integral do sistema olfactivo principal, não é o principal nem sequer o maior alvo das projecções do bolbo olfactivo. Como se sabe, estas projecções atingem os diversos componentes do córtex olfactivo primário, do qual o nLOT não é considerado

por muitos autores parte integrante (para revisão, ver Ennis et al., 2015). Face a estes dados e porque, nos roedores, a activação do sistema olfactivo principal por odores voláteis parece ser importante para o comportamento social e acasalamento (para revisão, ver Petrulis, 2013; Baum e Cherry, 2015), avaliámos ainda se os ratos lesados demonstravam atracção pelos odores voláteis provenientes da urina de fêmeas receptivas. Verificámos que tal não acontecia, já que a percentagem de tempo gasto pelos ratos lesados no canto do *open-field* onde a urina proveniente de fêmeas receptivas estava colocada não diferiu do despendido nos restantes cantos, onde se encontrava depositada urina ou de fêmeas não receptivas ou de machos ou água. O conjunto destes dados permitiu-nos concluir que as lesões do nLOT alteram significativamente a actividade sistema olfactivo principal, sem que, no entanto, permita vislumbrar por que motivo a destruição de apenas um alvo de projecção do bolbo olfactivo provoca alterações tão exuberantes nas funções que lhe estão cometidas. Este é, sem dúvida, um tema que merece estudo mais aprofundado.

Sabe-se que alguns odores despertam comportamentos inatos que são essenciais para a sobrevivência das espécies (Ennis et al., 2015), nomeadamente respostas aversivas aos odores de predadores (para revisão, ver Canteras et al., 2015). Foi demonstrado que alguns núcleos/áreas do grupo cortical da amígdala (Wernecke et al., 2015), e em particular o núcleo cortical póstero-lateral (Root et al., 2014), que recebem projecções topograficamente separadas do bolbo olfactivo principal (Pro-Sistiaga et al., 2007; Sosulski et al., 2011), são essenciais para a exibição deste comportamento. Porque o nLOT também pertence a este grupo nuclear, analisámos ainda os efeitos da sua lesão nos comportamentos inatos de aversão a odores de predadores. Utilizámos odores de diversos predadores que se sabe activarem diferentes receptores específicos do neuroepitélio olfactivo ou receptores do órgão vomeronasal. Verificámos que nenhum destes odores desencadeava comportamentos aversivos, independentemente do receptor activado. Dado existirem trabalhos que demonstram que os odores aversivos e os atractivos activam diferentes subtipos de neurónios e diferentes regiões topográficas do núcleo cortical póstero-lateral (Root et al., 2014), também testámos um odorante (2PE) que é naturalmente atractivo. Também neste os ratos lesados não responderam com comportamento sugestivo de atracção. Estas observações sugerem que o nLOT poderá desempenhar papel importante nas respostas comportamentais a estes tipos de odores,

uma vez que não projecta para nenhum dos restantes núcleos corticais da amígdala que se sabem estar envolvidos nestes comportamentos.

Para além de receber aferências provenientes do bolbo olfactivo, o nLOT também recebe projecções do bolbo olfactivo acessório (Pro-Sistiaga et al., 2007; Gutiérrez-Castellanos et al., 2014), sendo actualmente reconhecido como uma das várias áreas cerebrais onde estes dois sistemas convergem. Sabe-se que a interacção física activa o sistema olfactivo acessório (revisto em Baum e Cherry, 2015; Hashikawa et al., 2016) e que este sistema é, em termos funcionais, complementar do sistema olfactivo principal. Por este motivo, analisámos também se as lesões do nLOT alterariam comportamentos específicos dos roedores machos nos quais a actividade de ambos os sistemas é complementar, designadamente o comportamento sexual e a agressividade. Verificámos que a fase pré-copulatória do comportamento sexual estava significativamente alterada e que estes ratos não exibiam *mounting*. Acresce que após lesão do nLOT, os ratos despendiam, ao contrário dos ratos controlo e *sham*, mais tempo em comportamentos defensivos do que agressivos quando um rato desconhecido era colocado nas suas gaiolas.

No seu conjunto, os estudos comportamentais realizados permitiram demonstrar que a integridade do nLOT é fundamental para a normalidade tanto da percepção e discriminação olfactiva como para a expressão de comportamentos mediados pelo olfacto.

A disfunção olfactiva associada ao envelhecimento acompanha-se de supressão do apetite, que resulta em perda ponderal, má-nutrição, distúrbios da imunidade e deterioração do estado geral (revisto em Seiberling e Conley, 2004; Hummel e Nordin, 2005; Boyce e Shone, 2006). Os dados obtidos no TRABALHO 1, onde também se analisaram os efeitos do envelhecimento na estrutura e em algumas características neuroquímicas do nLOT, demonstraram que este núcleo é vulnerável ao envelhecimento e que as alterações que nele ocorrem podem ajudar a compreender a disfunção olfactiva dos idosos.

Com efeito, verificou-se que o envelhecimento se acompanhava de diminuição significativa do volume da camada 3 do nLOT e de redução, de aproximadamente 14%, do número total de neurónios das camadas 2 e 3. Para além destas alterações, verificámos

existir redução significativa do número total de neurónios imunorreactivos para o NPY e o VIP, que pode traduzir ou morte neuronal ou tão-só redução da expressão dos neuropeptídeos para níveis inferiores aos detectáveis pelo método immunocitoquímico utilizado. Dado que tanto o NPY como o VIP são expressos por interneurónios GABAérgicos, a alteração da expressão destes neuropetídeos ou a diminuição do número destes interneurónios poderá ter repercussões sobre a actividade dos neurónios piramidais, que são glutamatérgicos, e, portanto, sobre a influência exercida pelo nLOT sobre as suas áreas-alvo.

Dado que o nLOT é ricamente inervado por fibras colinérgicas, e que é possível que o envelhecimento não-patológico possa resultar de disfunção colinérgica (revisto em Schliebs e Arendt, 2011), analisámos também se o envelhecimento se acompanharia de alterações da sua inervação colinérgica. A análise da densidade das varicosidades imunorreactivas para a *vesicular acetylcholine transporter* (VAChT) demonstrou que, no caso do nLOT, as alterações estruturais e neuroquímicas associadas ao envelhecimento não decorriam de alterações na inervação colinérgica do núcleo.

No seu conjunto, os dados obtidos permitem adicionar o nLOT ao conjunto das regiões relacionadas com o processamento olfactivo que ficam afectadas durante o envelhecimento normal, como é o caso do bolbo olfactivo, núcleo olfactivo anterior, tubérculo olfactivo, córtex piriforme, amígdala e córtex entorrinal (revisto em Doty e Kamath, 2014; Adjei et al., 2013; Mobley et al., 2014) e, assim, contribuir para demonstrar a particular vulnerabilidade do sistema olfactivo no decurso do envelhecimento.

O conjunto de estudos incluídos no TRABALHO 3 da dissertação permitiram demonstrar que o stresse crónico se acompanha de alterações significativas do olfacto e dos comportamentos a ele associados e, ainda, de alterações estruturais e neuroquímicas do nLOT. Utilizou-se para o efeito um modelo animal de stresse crónico variável, a que os ratos foram expostos durante 4 semanas. Alguns dos ratos foram ulteriormente mantidos em condições *standard* por um período adicional de 4-7 semanas (grupo recuperado).

Os dados obtidos demonstraram que o stresse crónico provoca acentuados défices olfactivos, designadamente incapacidade de detecção e de discriminação de odores

neutros e sociais, o mesmo se tendo verificado em relação à preferência por odores voláteis provenientes da urina de fêmeas receptivas e à capacidade de discriminação de odores aversivos e atractivos. Verificou-se também que estes efeitos não são prontamente revertidos após interrupção da exposição ao stresse, já que 4-7 semanas após cessação de exposição ao stresse a capacidade de detecção e de discriminação de odores ainda se encontrava comprometida. A análise dos comportamentos inatos de atracção e de aversão revelou que estes apenas se encontravam parcialmente recuperados, mesmo 6 semanas após o fim da exposição a stresse crónico. Tendo em consideração a importância, já antes discutida, dos sistemas olfactivos principal e acessório nestas funções e comportamentos, os dados obtidos mostram que o stresse crónico tem forte impacto na actividade destes sistemas e que os seus efeitos são prolongados dado que, 6-7 semanas após interrupção da exposição ao stresse, os seus efeitos estavam apenas parcialmente revertidos.

Ao contrário do observado nestas funções, os ratos submetidos a stresse crónico não apresentavam qualquer alteração do comportamento sexual ou da agressividade, apesar de os níveis circulantes de testosterona estarem significativamente reduzidos. Estes resultados não estão em sintonia com dados de outros autores (D'Aquila et al., 1994; Grønli et al., 2005; Wang et al., 2012), que demonstraram estar o comportamento sexual e a agressividade alterados nestas condições experimentais. É de salientar que nestes estudos os testes comportamentais foram realizados durante o período de exposição ao stresse e não após o final deste período, como no presente estudo. Analisados no seu conjunto, estes dados mostram que os efeitos do stresse crónico no comportamento sexual e na agressividade são de curta duração e desaparecem rapidamente após a cessação da exposição ao stress. Sugerem também que a actividade do sistema olfactivo acessório é rapidamente restabelecido caso este sistema seja activado pela investigação social.

Estudos estereológicos relativamente recentes mostraram que a exposição ao stresse durante um período de 4 semanas não altera a morfologia da maioria dos núcleos não-corticais da amígdala (Pêgo et al., 2008), embora exposições menos prolongadas conduzam a hipertrofia dendrítica na amígdala basolateral (Vyas et al., 2002). O estudo estereológico desenvolvido no TRABALHO 3 mostra que, ao contrário do que foi descrito para a maioria dos núcleos não-corticais da amígdala, o stresse crónico provoca alterações estruturais e neuroquímicas apreciáveis no nLOT. Especificamente, verificámos que o stresse crónico causa hipertrofia somática dos neurónios do nLOT e atrofia dos interneurónios imunorreactivos para a PV, CB e CR, o que sugere que apenas os

neurónios de projecção, que são glutamatérgicos, se encontram hipertrofiados em ratos stressados. A diminuição do volume dos corpos celulares dos interneurónios acompanhou-se de redução significativa dos níveis de GABA no nLOT, o que sugere que a hipertrofia, e possivelmente hiperactividade, dos neurónios de projecção do nLOT, resulta da diminuição do *status* inibitório local. Também estes efeitos parecem ser irreversíveis, ou pelo menos duradouros, porque após 7 semanas de recuperação, os neurónios do nLOT permaneceram hipertrofiados e os interneurónios imunorreactivos para CB e CR atrofiados, tendo apenas os interneurónios produtores de PV recuperado o seu volume normal. Acresce que os níveis de GABA do nLOT permaneceram significativamente reduzidos relativamente aos dos controlos.

Em suma, o conjunto dos estudos que constam do TRABALHO 3 demonstra, tanto quanto é do nosso conhecimento, pela primeira vez que o stresse crónico conduz a distúrbios olfactivos graves e prolongados e que estes efeitos se acompanham de alterações estruturais e neuroquímicas num núcleo da divisão cortical da amígdala, o nLOT.

CONCLUSÕES

O conjunto dos resultados reportados nos trabalhos que constam da presente dissertação permitiram:

1. Caracterizar, através da aplicação de técnicas estereológicas, os parâmetros morfométricos básicos do nLOT, designadamente o seu volume global e o das suas camadas, o número total das suas populações celulares, o volume neuronal médio e a densidade numérica das varicosidades colinérgicas. Estes dados permitem ser colacionados com outros estudos experimentais envolvendo este núcleo.

2. Demonstrar que o corolário das lesões excitotóxicas do nLOT de ratos machos sexualmente inexperientes foi insigne, particularmente no referente à perda total do olfacto com incapacidade de detecção e discriminação de odores. Este facto obstou os comportamentos dependentes do olfacto que são críticos para a sobrevivência e reprodução. Não sendo cognoscíveis as razões para a magnitude e severidade destes efeitos, apenas podemos especular que, independentemente dos atributos funcionais de cada componente do sistema olfactivo, a normalidade das funções olfactivas e dos comportamentos dependentes do olfacto parecem depender da integridade de todos os seus componentes.

3. Mostrar que o nLOT apresenta perda neuronal e diminuição da expressão neuropeptídica com o envelhecimento, o que está de acordo com o observado por outros autores noutras áreas do sistema olfactivo. Estas alterações morfológicas e neuroquímicas podem contribuir para a deterioração funcional do córtex olfactivo e da percepção dos odores que se verifica com a vetustez.

4. Demonstrar que o stresse crónico origina alterações estruturais prolongadas a nível do nLOT, designadamente hipertrofia somática dos neurónios piramidais glutamatérgicos, atrofia da população GABAérgica local e redução da concentração de GABA no núcleo. O aumento do volume neuronal parece dever-se à redução da influência inibitória exercida pelos interneurónios locais, e manteve-se de forma diuturna.

5. Evidenciar que o stresse crónico origina défices olfactivos importantes, designadamente incapacidade de detecção e discriminação de odores e total inépcia de detecção de odores de predadores. Sendo o nLOT uma estrutura para onde convergem informações veiculadas pelos sistemas olfactivos principal e acessório, a sua disfunção pode contribuir para os défices olfactivos associados ao stresse crónico e que podem colocar em risco a sobrevivência da espécie.

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RESUMO

Os estudos incluídos nesta dissertação têm como objectivo principal caracterizar, do ponto de vista morfológico e neuroquímico, o núcleo do tracto olfactivo lateral (nLOT), avaliar as suas atribuições funcionais e comportamentais e averiguar a sua vulnerabilidade ao envelhecimento e ao stresse crónico.

Recorreu-se a vários modelos experimentais, tais como lesões excitotóxicas bilaterais do nLOT, o envelhecimento e o stresse crónico, analisando-se os respectivos efeitos neste núcleo através de estudos comportamentais e aplicação de métodos estereológicos. Estimou-se o volume global do núcleo e das suas camadas, o número de neurónios das suas várias populações celulares por camada, o volume médio por camada dos corpos celulares desses mesmos neurónios e, ainda, a densidade das varicosidades colinérgicas no nLOT e os níveis de GABA deste núcleo.

Verificou-se que o efeito das lesões excitotóxicas do nLOT de ratos machos sexualmente inexperientes foi insigne, particularmente a perda total do olfacto com a incapacidade de detecção e discriminação de odores. Este facto embargou os comportamentos dependentes do olfacto que são críticos para a sobrevivência e reprodução, nomeadamente o comportamento aversivo a odores de predadores e o comportamento agressivo relativamente a ratos intrusos. Os ratos lesados também não exibiam comportamentos sugestivos da preferência pelos odores voláteis provenientes da urina de fêmeas receptivas, e tinham menor comportamento de investigação social e de perseguição das fêmeas e completa ausência de *mounting*. Constatou-se, também, que os ratos lesados tinham níveis normais de testosterona, não apresentavam comportamentos sugestivos de ansiedade ou de depressão, e tinham preservação da aprendizagem e memória espacial, e da aquisição do medo condicionado. Não sendo ainda compreensíveis as razões para a magnitude e severidade destes efeitos, apenas podemos especular que, independentemente dos atributos funcionais de cada componente do sistema olfactivo, a normalidade das funções olfactivas e dos comportamentos dependentes do olfacto parecem depender da integridade de todos os seus componentes.

Mostrou-se que o nLOT é uma estrutura vulnerável ao envelhecimento. Efectivamente, o nLOT apresenta perda neuronal nas suas camadas celulares e diminuição do número de neurónios imunorreactivos para VIP e NPY. Não foram detectadas alterações no volume neuronal e na densidade das varicosidades colinérgicas. Assim, verificou-se que o nLOT se comporta tal como outras áreas encefálicas em que está demonstrado existir perda neuronal e diminuição da expressão de neuropeptídeos bem como manutenção da

inervação colinérgica no decurso do envelhecimento normal. A diminuição observada na expressão de neuropeptídeos não se acompanha de concomitante redução na inervação colinérgica, o que mostra não existir neste núcleo, e ao contrário de outras áreas encefálicas, uma relação directa entre a expressão destes neuropeptídeos e o número de varicosidades colinérgicas.

O modelo experimental de stresse crónico demonstrou que, além de causar comportamento de ansiedade e depressão, originou défices olfactivos importantes com incapacidade de detectar e discriminar odores e total inépcia de detectar odores de predadores. Os ratos submetidos a stresse crónico também não manifestavam comportamentos de atracção relativamente aos odores voláteis provenientes da urina de fêmeas receptivas, nem comportamentos de atracção ou evicção relativamente a odores agradáveis e odores de predadores, respectivamente. Estes efeitos foram acompanhados de hipertrofia somática dos neurónios piramidais glutamatérgicos, atrofia da população GABAérgica local, nomeadamente dos interneurónios produtores de parvalbumina, calbindina e calretitina, e menor concentração de GABA no nLOT. Neste modelo experimental não se verificaram alterações do comportamento sexual e de agressividade. Os danos induzidos pelo stresse persistiram, de forma relativamente inalterada, após 4-7 semanas de ausência de exposição a stresse, o que sugere que os efeitos do stresse crónico na estrutura e função do sistema olfactivo efeitos são prolongados e, potencialmente, irreversíveis.

ABSTRACT

The main objective of the studies included in this dissertation is to characterize the morphology and neurochemistry of the nucleus of the lateral olfactory tract (nLOT), to evaluate its functional and behavioral attributions and to assess its vulnerability to aging and chronic stress.

We have used several experimental models, such as bilateral excitotoxic lesions of the nLOT, aging and chronic stress to analyze their effects in this nucleus through the use of behavioral testing and application of stereological methods. The overall volume of the nucleus and its layers, the number of neurons of its various cell populations per layer, the mean somatic volume of these same neurons per layer, the density of the cholinergic varicosities in the nLOT and the levels of GABA of this nucleus were evaluated.

The functional and behavioral effects of the excitotoxic lesions on the nLOT in sexually inexperienced male rats were dramatic, including total loss of the sense of smell with inability to detect and discriminate odors. This has constrained olfactory-dependent behaviors that are critical to survival and reproduction, including predatory odor aversive behavior and aggressive behavior towards intruding rats. The injured rats also did not exhibit preference for volatile odors from the urine of receptive females, had lower social behavior and female pursuit, and complete absence of mounting. It was also found that the injured rats had normal levels of testosterone, did not show depression or anxiety-like behaviors, and had preserved spatial learning and memory, and conditioned fear acquisition. We do not know the reasons for the magnitude and severity of these effects and, therefore, we can only speculate that, regardless of the functional attributes of each component of the olfactory system, the normality of olfactory functions and olfactory dependent behaviors seem to depend on the integrity of all its components.

We also showed that the nLOT is vulnerable to aging. Indeed, nLOT exhibits neuronal loss in its cell layers and decreased numbers of neurons immunoreactive for VIP and NPY. No changes were detected in the mean neuronal volume or in the density of cholinergic varicosities. Thus, we found that the nLOT displays age-related changes similar to those described for other regions of the brain where neuronal loss, decreased neuropeptide expression and maintenance of the cholinergic innervation have been demonstrated in the course of normal aging. The observed decrease in the expression of neuropeptides was not accompanied by a concomitant reduction in the cholinergic innervation, which shows that, unlike other brain regions, there is no direct relation

between the expression of these neuropeptides and the number of cholinergic varicosities in this nucleus.

The experimental model of chronic stress, in addition to causing depression and anxiety-like behaviors, also induced important olfactory deficits, with inability to detect and discriminate between odors and total incapacity to detect odors from predators. They also did not exhibit attraction towards volatile odors from the urine of receptive females, nor did they exhibit attraction or aversion towards appetitive odors or predator odors, respectively. These effects were accompanied by somatic hypertrophy of glutamatergic pyramidal neurons, atrophy of the local GABAergic population, in particular interneurons producing parvalbumin, calbindin and calretinin, and lower local concentrations of GABA. There were no changes in sexual behavior and aggression. The stress-induced damage persisted without major alterations for 4-7 weeks after the last stress exposure, suggesting that the effects of chronic stress on the structure and function of the olfactory system are long-lasting and potentially irreversible.